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ORIGINAL RESEARCH

RETRACTED ARTICLE: *Let-7c-5p* Inhibits Cell Proliferation and Migration and Promotes Apoptosis via the CTHRC1/AKT/ERK Pathway in Esophageal Squamous Cell Carcinoma

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Purpose: *Let-7c-5p* has been identified as a tumor suppressor in various malignancies; however, its function and mechanism in esophageal squamous cell carcinoma (ESCC) remain unclear. Here, we explored the role and potential molecular mechanism of *let-7c-5p* in ESCC.
Materials and Methods: mRNA and protein expression levels were detected by quantitative real time-polymerase chain reaction (qRT-PCR) and Western blotting. The cell counting kit-8 (CCK-8) assay was used to assess cell proliferation. Flow cytometry analysis was used to detect cell apoptosis, and cell migration was measured by wound healing assay and Transwell assays. The dual-luciferase reporter assay was used to verify the targeting relationship between *let-7c-5p* and CTHRC1. The tumor xenograft model was constructed to further verify the effect of *let-7c-5p* on the growth of ESCC in vivo.
Results: We found that *let-7c-5p* expression was downregulated in ESCC tissue and cell lines, and its reduced expression was correlated with TNM staging and lymph node metastasis. Next, we found that *let-7c-5p* can be used to discriminate ESCC patients from normal controls subjects by receiver operating characteristic (ROC) curve analysis. Subsequently, we observed that *let-7c-5p* overexpression inhibited proliferation and migration and promoted apoptosis, while *let-7c-5p* down-regulation promoted proliferation and migration and inhibited apoptosis of TE-1 and KYSE150 cells. Furthermore, *let-7c-5p* overexpression inhibited tumor growth, while *let-7c-5p* inhibition promoted tumor growth in xenograft models. In addition, we confirmed that CTHRC1 was a direct target gene of *let-7c-5p*. Then, we found that *let-7c-5p* level was negatively correlated with CTHRC1 and negatively regulated expression of CTHRC1 in ESCC. Moreover, we confirmed that *let-7c-5p* upregulation significantly reduced the phosphorylation of AKT and ERK by directly inhibiting CTHRC1, while *let-7c-5p* downregulation showed the opposite effect.
Conclusion: Our findings indicate that *let-7c-5p* is markedly downregulated in ESCC and suppresses proliferation and migration and promotes apoptosis of ESCC cells by inhibiting the AKT and ERK signaling pathways through negatively regulating CTHRC1. Therefore, these results suggest that *let-7c-5p* may represent a novel biomarker and therapeutic target for ESCC.

Keywords: *let-7c-5p*, ESCC, CTHRC1, proliferation, migration, apoptosis

Introduction

As a highly aggressive malignant tumor, esophageal cancer (EC) is the seventh most common cancer in the world (3.2% of total) and is the sixth leading cause of

cancer-related mortality (5.3% of total).[1] Esophageal squamous cell carcinoma (ESCC) is the most common histological subtype of EC, accounting for more than 90% of all cases.[2] It is characterized by invasiveness, recurrence and metastasis, and the overall five-year survival rate is only 15% to 25%.[3] Although diagnostic technologies and treatment methods have continuously advanced, most patients are diagnosed at an advanced stage, and the overall five-year survival rate is still meager.[4] In recent years, molecular targeted therapy has made progress with respect to the diagnosis and treatment of EC, for example, by use of VEGF, EGFR and HER2.[5–8] Therefore, it is crucial to explore the potential mechanisms of ESCC at the molecular level. To enhance earlier detection of ESCC patients, we urgently need to identify additional noninvasive molecular biomarkers with high sensitivity and specificity for ESCC.

MicroRNAs (miRNAs) are a type of endogenous, short noncoding RNA that contains approximately 21–24 nucleotides. Mature miRNAs generally negatively regulate expression of their target genes by binding to the 3′-untranslated region (3′-UTR) of target mRNAs, leading to mRNA degradation or translational inhibition.[9,10] Recent studies have reported that miRNAs regulate approximately 33% of the expression of various human genes.[11] Increasing evidence has shown that miRNAs play a significant role in carcinogenesis with respect to cell proliferation, differentiation, cycle, apoptosis, migration, invasion, metastasis and angiogenesis.[12–14] In addition, numerous studies have reported that some miRNAs act as tumor promoters or suppressors in human tumors depending on the regulated tumor forms and the specific targets involved.[15–17] The miRNA *let-7*, one of the earliest identified human miRNAs, participates in various biological processes such as cell proliferation, differentiation, apoptosis, hormone secretion, metabolism, immune regulation and tumorigenesis. Recent studies have found that *let-7c-5p* is significantly downregulated in a variety of human malignant tumors, including head and neck squamous cell carcinoma,[18] non-small cell lung cancer,[19] hepatocellular carcinoma,[20] colorectal cancer,[21] ovarian carcinoma[22] and glioma.[23] Importantly, based on the results of unsupervised hierarchical clustering in previous literature, it was found that *let-7c* was downregulated in ESCC tissues.[24,25] However, the biological function and molecular mechanism of *let-7c-5p* in ESCC remain unclear. We hypothesized that *let-7c-5p* might represent a promising biomarker of ESCC.

Collagen triple helix repeat containing 1 (CTHRC1) is a type of secreted extracellular matrix glycoprotein of approximately 28 kDa with a C-terminal globular domain, a 36-amino acid short collagen triple helix repeat sequence, and an N-terminal signal peptide for extracellular secretion peptide.[26,27] It was initially identified in the screening of differentially expressed sequences for balloon injury and normal rat arteries.[28] A large number of studies have shown that CTHRC1 plays an oncogenic role in various tumors, and its high expression indicates poor prognosis.[29–31] Furthermore, a recent study found that CTHRC1, a tumor promoting factor, was significantly upregulated in ESCC tissues and was closely correlated with TNM staging, lymph node metastasis and poor prognosis.[32] Nevertheless, the regulatory relationship between *let-7c-5p* and CTHRC1 in ESCC and the potential mechanism require further exploration.

The goal of this study was to explore the role and potential molecular mechanism of *let-7c-5p* in the occurrence and development of ESCC. Specifically, we assessed expression of *let-7c-5p* in ESCC by qRT-PCR. We further analyzed the relationship between *let-7c-5p* and clinicopathological characteristics. Then, we conducted ROC curve analysis on *let-7c-5p*. Moreover, we evaluated tumor-associated phenotypes, including cell proliferation, apoptosis, and migration by transfecting *let-7c-5p* mimics and inhibitors into TE-1 and KYSE150 cells. Next, bioinformatics analysis predicted that CTHRC1 might be a potential target gene of *let-7c-5p*, which was further verified by a dual-luciferase report experiment. Furthermore, we verified the effect of *let-7c-5p* on the progression of ESCC in vivo. Finally, we explored the possible molecular mechanism of *let-7c-5p* by Western blot analysis. Because molecular biomarkers play an essential role in the clinical diagnosis of ESCC, our current research on *let-7c-5p* has important implications for early diagnosis and treatment of ESCC.

Materials and Methods

Tissues and Cell Lines

Fifty ESCC tissues and matched adjacent noncancerous tissues were collected from patients with esophageal cancer who had undergone surgery in the Affiliated Hospital of Southwest Medical University. All clinical tissues were collected after obtaining informed consent from the subjects. This study was approved by the clinical trial ethics

committee of the Affiliated Hospital of Southwest Medical University (No. KY2020103). ESCC tissues were collected and immediately placed in liquid nitrogen and stored at −80°C. The human ESCC cell lines, TE-1, TE-10, TE-11, KYSE140, and KYSE150, the normal esophageal epithelial cell line Het-1A and the human embryonic kidney cell line HEK-293T were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China), 10kU/mL penicillin and 10mg/mL streptomycin (Beyotime, Shanghai, China). Cells were incubated in a 5% CO2 humidified incubator at 37°C.

Cell Transfection

Let-7c-5p mimics, inhibitor, corresponding negative control vectors, CTHRC1 siRNA (siCTHRC1) and the negative control vector were purchased from RiboBio (Guangzhou, China). Transfection was performed with Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer's protocol. The day before transfection, cells were collected and seeded into well plates at a density of 60–70%. Then, 6–8 hours after transfection, the medium was replaced with RPMI-1640 containing 10% FBS (without antibiotics). The sequences of *let-7c-5p* mimics, inhibitor and siCTHRC1 are shown in Supplementary Table 1.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from ESCC tissues and cells using TRIzol reagent (Tiangen, Beijing, China) following the manufacturer's instructions and then quantified using NanoDrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). For *let-7c-5p*, 500ng RNA were reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA kit (Tiangen, Beijing, China). PCR amplifications were performed using SYBR Green Premix qPCR Master Mix (Tiangen, Beijing, China). For CTHRC1, 800ng RNA were reverse transcribed into cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) and qRT-PCR was performed with SYBR-Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). PCR amplifications and detection were performed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA). All primers were purchased from RiboBio (Guangzhou, China). Primer sequences are shown in Supplementary Table 2. U6 was used as an endogenous control for *let-7c-5p*, while GAPDH was an internal control for CTHRC1. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression.

Cell Counting Kit-8 Assay (CCK-8)

The CCK-8 assay was used to determine cell growth in response to *let-7c-5p* mimics or inhibitor transfected ESCC cells. Transfected cells were plated into 96-well plates at a density of $4x10^3$ cells per well and cultured in a 5% CO2 humidified incubator at 37°C for 24, 48, 72 or 96 hours. Ten microliters of CCK-8 reagent were then added and cultured for another 2 hours. Cell optical density was subsequently measured at 450nm by a full-wavelength spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Flow Cytometry Analysis to Assess Apoptosis

Flow cytometry was used to detect apoptosis using an Annexin-V/PI kit (BD Biosciences, New Jersey, USA). Forty-eight hours after transfection, cells were collected and washed once with cold phosphate-buffered saline (PBS) and then washed once with 1×binding buffer. Then, the cell concentration was adjusted to $1x10^6$/mL. Five microliters of Annexin-V and PI were added to every 100 µL of resuspended cells solution and then incubated for 15 min at room temperature in the dark. Cells were subjected to flow cytometry (BD Biosciences, New Jersey, USA), and data were analyzed by the FlowJo (NIH, Bethesda, Massachusetts, USA).

Wound Healing Assay

Artificial wounds were created to observe the migration of ESCC cells. Transfected cells were seeded in 12-well plates at a density of $1x10^5$ cells per well and cultured in a humidified incubator with 5% CO_2 at 37°C. After cells reached 90% confluence, a 10 µL sterile pipette tip was used to create an artificial wound on the plates. Cells were washed 3 times with PBS, cultured in fresh RIPM-1640 medium with 2% FBS, and incubated for 24 hours. Images were captured using an inverted phase-contrast microscope at 0 and 24 hours. Cell mobility was assessed by measuring the distance between the boundaries of migrating cells. The migrated wound area was calculated using ImageJ software (NIH, Bethesda, Massachusetts, USA).

Transwell Migration Assay

The Transwell migration assay was used to detect the migration ability of ESCC cells using a Transwell apparatus (Costar, Corning Inc., USA) with an 8 μm porous polycarbonate membrane slide chamber. Transfected cells were collected and resuspended in serum-free RPMI 1640, and $6x10^4$ cells were added to the upper chamber. Next, 600 μL of fresh RPMI 1640 media containing 20% FBS were added to the lower chamber. After 48 hours of incubation, cells on the upper surface of the chamber were gently wiped off with a wet cotton swab. Then, remaining cells were washed 3 times in PBS. Cells migrating to the bottom surface of the chamber were fixed in 4% paraformaldehyde for half an hour, stained with 1% crystal violet for 3 minutes, washed 3 times with PBS, and dried naturally at room temperature. Images were captured using an inverted phase-contrast microscope. Cell migration rate was evaluated by counting the number of migrated cells. Migrated cells were calculated using ImageJ software (NIH, Bethesda, Massachusetts, USA).

Prediction of miRNA Target Genes

In this study, three independent databases were used to predict potential targets of *let-7c-5p*, including TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org/) and miRanda (www.microrna.org/microrna/home.do).

Dual-Luciferase Reporter Assay

A dual-luciferase reporter assay was used to investigate whether *let-7c-5p* binds to the 3′-UTR of CTHRC1. The wild type (WT) and mutant (MUT) 3′-UTRs of CTHRC1 were synthesized by RiboBio Biotechnology (Wuhan, China) and then inserted into the pGL6 basic vector with BamHI and HindIII restriction sites. The sequences of the plasmid vectors are shown in Supplementary Table 3. HEK-293T cells were cultured in 96-well plates at a density of 50–70%. Forty-eight hours after transfection, cells were collected and luciferase activity was detected with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In this experiment, renilla luciferase activity was used as the reporter gene, and firefly luciferase was used as the reference gene.

Western Blot Analysis

Total protein was extracted from ESCC tissues and cells using cold RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations were determined with the BCA protein determination kit (Beyotime, Shanghai, China). An equal concentration of protein samples was added to each sample well and separated by 10% SDS-PAGE, and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in Tris-buffered saline solution with 0.1% Tween-20 (TBST) containing 5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies were incubated overnight on a shaker at 4°C. The primary antibodies used in this experiment are as follows: rabbit anti-CTHRC1 (1:2000, ab256458, Abcam, Cambridge, MA, USA), rabbit anti-ERK1/2 (1:2000, #4370, Cell Signaling, Danvers, MA, USA), rabbit anti-p-ERK1/2 (1:2000, #8544, Cell Signaling, Danvers, MA, USA), rabbit anti-AKT (1:2000, #9272, Cell Signaling, Danvers, MA, USA), rabbit anti-p-AKT (1:2000, #9271, Cell Signaling, Danvers, MA, USA) and rabbit anti-GAPDH (1:10,000, #MB001H, Bioworld, Beijing, China). After washing 5 times in TBST, blots were incubated with secondary antibody (1:3000, Beyotime, Shanghai, China) for 1 hour at room temperature. Blots were visualized using the ECL Luminescent Solution (Millipore, Billerica, MA, USA). GAPDH was used as an internal control. Protein bands were analyzed using ImageJ software (NIH, Bethesda, MA, USA).

Xenograft Tumor Model

Male BALB/c-nu mice (4–5 weeks of age) were purchased from Beijing Sibefu Biotechnology Co., Ltd. (Beijing, China). With the permission of the Ethics Committee of the Affiliated Hospital of Southwest Medical University, the entire animal experiment was carried out in strict accordance with animal procedures. *Let-7c-5p* agomir, agomir-NC, antagomir, and antagomir-NC were purchased from RiboBio (Guangzhou, China). We inoculated 200μL of PBS (containing 1×10^7 TE-1 cells) into the subcutaneous tissues of the bilateral armpits of each nude mice, and measured the size of the tumor every 3 days for 3 weeks. Finally, the nude mice were euthanized, and the tumor tissues were removed for Western blot analysis. The tumor volume (V) was measured by the formula: V (mm^3) = (1/2) × length × $width^2$.

Statistical Analysis

All experiments were performed in triplicate. SPSS version 25.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All results are expressed as the

mean ± standard deviation (SD). Pearson's $\chi 2$ test was used to investigate the relationship between *let-7c-5p* and clinicopathological characteristics of ESCC tissues. Pearson's correlation analysis was used to examine the correlation between *let-7c-5p* and CTHRC1. Statistical differences between the two groups were analyzed by Student's *t*-test. One-way analysis of variance was used for comparisons among multiple groups. Values of $p<0.01$ or $p<0.05$ were considered statistically significant.

Results

Expression of Let-7c-5p in ESCC and Its Relationship with Clinicopathological Characteristics

To explore the potential clinicopathological significance of *let-7c-5p* in ESCC, we assessed expression of *let-7c-5p* in ESCC tissues and cell lines by qRT-PCR. Intriguingly, we found that expression of *let-7c-5p* was significantly downregulated in 50 pairs of ESCC tissues compared to adjacent nontumor tissue (Figure 1A). Similarly, we confirmed that *let-7c-5p* expression was markedly decreased in five ESCC cell lines (TE-1, TE-10, TE-11, KYSE140 and KYSE150) compared to normal esophageal epithelial cells, HEEC1A (Figure 1B). Next, we further analyzed the correlation between *let-7c-5p* and patient clinicopathological factors in ESCC. Importantly, we observed that lower expression of *let-7c-5p* was associated with advanced clinical stage (Figure 1C, Table 1, P<0.05) and lymph node metastasis (Figure 1D, Table 1, P<0.05); however, there was no significant correlation with gender, age, smoking status or tumor size (Table 1, P>0.05). Due to the reduced expression of *let-7c-5p* in TE-1 and KYSE150 cells, we conducted subsequent experiments in these two cell lines. These findings indicated that reduced expression of *let-7c-5p* might be predominently related to the underlying pathogenesis of ESCC.

Evaluation of Let-7c-5p as a Potential Biomarker of ESCC

We examined the utility of *let-7c-5p* as a potential biomarker for ESCC diagnosis by receiver operating characteristic (ROC) curve analysis. As shown in Figure 1E, the area under the curve (AUC) value of *let-7c-5p* in ESCC tissues was 0.81 [95% confidence interval (CI) = 0.727–0.894, P<0.0001]. The sensitivity and specificity at the cutoff value of 0.528 were 74% and 79%, respectively. These statistics indicated that expression of *let-7c-5p* successfully discriminates ESCC patients from normal control groups. Further, we evaluated the ability of *let-7c-5p* to screen early stage and late stage patients from controls by ROC curve analysis. Notably, the AUC of *let-7c-5p* in early stage ESCC tissues was 0.72 (95% CI = 0.589–-0.855, P = 0.0044) and at a cutoff value of 0.44, the optimal sensitivity and specificity were 80% and 64%, respectively. The AUC of *let-7c-5p* in advanced stage ESCC tissues was 0.88 (95% CI = 0.785–0.981, P<0.0001) and at a cutoff value of 0.68, the optimal sensitivity and specificity were 92% and 86%, respectively (Figure 1F). These results revealed that *let-7c-5p* may represent a valuable biomarker in both early and late stages of ESCC.

Let-7c-5p Suppresses Proliferation and Promotes Apoptosis in ESCC Cells

We explored the effect of *let-7c-5p* on proliferation and apoptosis of ESCC cells by CCK-8 assay and flow cytometry analysis. First, we detected the transfection efficiency of TE-1 and KYSE150 cells in response to transfection with *let-7c-5p* mimics or inhibitor by qRT-qPCR. As a result, compared to the control group, *let-7c-5p* was significantly upregulated after transfection with *let-7c-5p* mimics but was significantly downregulated after transfection with the *let-7c-5p* inhibitor in TE-1 and KYSE150 cells (Figure 2A). These findings indicate that *let-7c-5p* mimics or inhibitor effectively increase or decrease levels of *let-7c-5p*, respectively. Next, we evaluated changes in cell proliferation through the CCK-8 assay. Results showed that overexpression of *let-7c-5p* using its mimics significantly inhibited cell proliferation (Figure 2B). In contrast, its inhibition successfully promoted cell proliferation (Figure 2C). Moreover, results of flow cytometry analysis indicated that overexpression of *let-7c-5p* increased apoptosis (Figure 3A and B), inversely, inhibition of *let-7c-5p* reduced apoptosis (Figure 3C and D). Therefore, these results suggest that *let-7c-5p* inhibits proliferation activity and promotes apoptosis in ESCC cells.

Let-7c-5p Suppresses Migration in ESCC Cells

We used the wound healing assay and Transwell assay to explore the effects of *let-7c-5p* on cell migration. In the wound healing assay, we found that overexpression of *let-7c-5p* significantly inhibited the migration behavior of

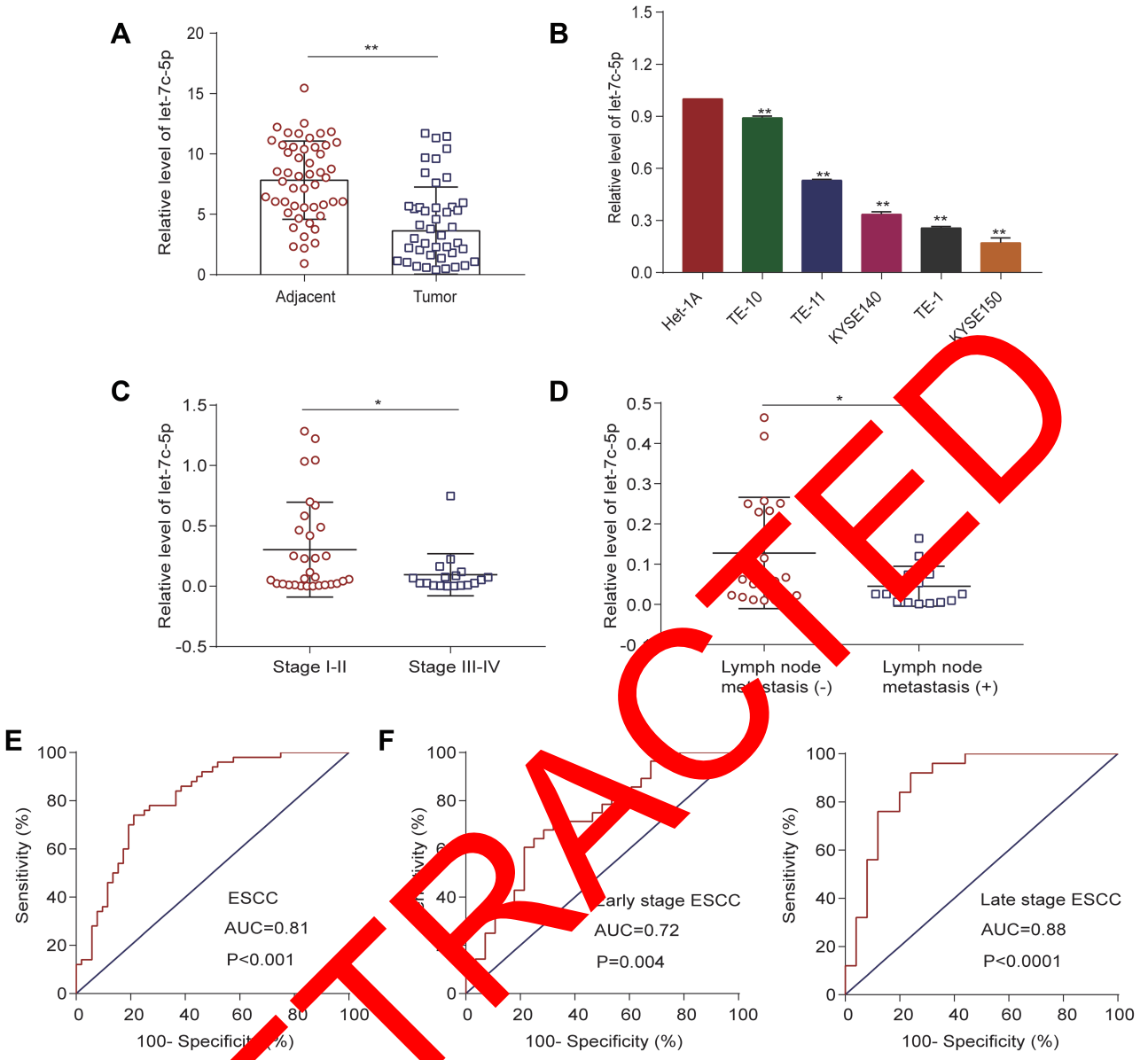

Figure 1 Expression of *let-7c-5p* in ESCC and its clinical significance. (**A**) Reduced mRNA expression of *let-7c-5p* in 50 pairs of ESCC tissues compared to adjacent normal tissues by qRT-PCR. (**B**) Decreased mRNA expression of *let-7c-5p* in five ESCC cell lines (TE-1, TE-10, TE-11, KYSE140 and KYSE150) compared to normal esophageal epithelial Het-1A cells by qRT-PCR. (**C**) Reduced mRNA expression of *let-7c-5p* in late stage ESCC tissues compared to early stage ESCC tissues by qRT-PCR. (**D**) Attenuated mRNA expression of *let-7c-5p* in ESCC tissues with lymph node metastasis compared to those without metastasis by qRT-PCR. (**E**) ROC curve analysis of the *let-7c-5p* assay ratio for discrimination between ESCC patients and normal controls. (**F**) Comparison of the diagnostic ability of *let-7c-5p* in early stage and late stage ESCC by ROC curve analysis. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. *p < 0.05 and **p < 0.01.

TE-1 and KYSE150 cells compared to the control group. In contrast, inhibition of *let-7c-5p* significantly promoted cell migration (Figure 4A and B). Moreover, we confirmed by the Transwell assay that *let-7c-5p* overexpression significantly inhibited cell migration compared to the control group, while *let-7c-5p* inhibition significantly promoted cell migration (Figure 4C and D). These results indicate that *let-7c-5p* inhibits the migration of ESCC cells.

CTHRC1 is a Direct Target of Let-7c-5p

To further explore the molecular mechanism of *let-7c-5p* effects, bioinformatics prediction analysis confirmed that CTHRC1 is a potential target of *let-7c-5p*, and its 3′-UTR contains a putative *let-7c-5p* binding site. CTHRC1's wild type 3′-UTR (pGL6-miR-CTHRC1-WT) contained the predicted *let-7c-5p* target site, while CTHRC1's mutant 3′-UTR (pGL6-miR-CTHRC1-Mut) lacked the *let-7c-5p* binding site (Figure 5A). To verify

Table 1 Association Between *Let-7c-5p* Expression and Clinicopathological Features of ESCC

Characteristics	Cases	*Let-7c-5p* Expression		P-value
		Low	High	
Sex				0.308
Male	33	16	17	
Female	3	2	1	
Age (years)				0.444
<60	12	7	5	
≥60	24	11	13	
Smoking status				0.117
No	5	4	1	
Yes	31	14	17	
Tumor size (cm)				0.312
<3	4	3	1	
≥3	32	15	17	
TNM stage				0.031*
I–II	21	10	11	
III–IV	15	8	7	
Lymph node metastasis				0.029*
Negative	22	10	12	
Positive	14	8	6	

Note: *Statistically significant.
Abbreviations: ESCC, esophageal squamous cell carcinoma; TNM, tumor node-metastasis.

bioinformatics predictions, we used the dual-luciferase reporter assay to investigate whether *let-7c-5p* directly targets CTHRC1. HEK-293T cells were co-transfected with pGL6-miR-CTHRC1-WT or pGL6-miR-CTHRC1-Mut vector and *let-7c-5p* mimics. Results demonstrated that *let-7c-5p* overexpression significantly reduced luciferase activity in CTHRC1 wild-type (WT) 3′-UTRs, compared to the negative control group. In contrast, in the absence of the *let-7c-5p* binding site, the inhibitory effect was significantly attenuated (Figure 5B). Next, to further explore the regulation of *let-7c-5p* on CTHRC1 expression, we found that overexpression of *let-7c-5p* significantly inhibited mRNA expression of CTHRC1 (Figure 5C), while inhibition of *let-7c-5p* restored CTHRC1 expression by qRT-PCR (Figure 5D). Moreover, compared to the control group, overexpression of *let-7c-5p* significantly inhibited protein levels of CTHRC1, while *let-7c-5p* inhibition restored protein levels of CTHRC1 by Western blot (Figure 5E and F). These results indicate that CTHRC1 is a direct target of *let-7c-5p* and *let-7c-5p* regulates expression of CTHRC1 by binding to its 3′UTR.

Expression of CTHRC1 in ESCC and Its Correlation with Let-7c-5p

We detected the expression of CTHRC1 in ESCC tissues and their adjacent normal tissues by qRT-PCR and Western blot. Significantly, we observed higher mRNA expression of CTHRC1 in ESCC tissues compared to adjacent normal tissues (Figure 6A). Then, we confirmed higher mRNA expression of CTHRC1 in ESCC cell lines compared to Het-1A (Figure 6B). Next, compared to adjacent normal tissues, protein levels of CTHRC1 in ESCC tissues were significantly upregulated (Figure 6C). Consistently, compared to those in Het-1A cells, the protein levels of CTHRC1 in TE-1 and KYSE150 cells were also significantly increased (Figure 6D). Moreover, we demonstrated that expression of *let-7c-5p* was significantly negatively correlated with CTHRC1 expression (Figure 6E, r =−0.418, p=0.009). These findings indicate that CTHRC1 is upregulated in ESCC and is negatively correlated with *let-7c-5p*.

Let-7c-5p Suppresses AKT and ERK Signaling Pathways in vitro

Since studies have reported that CTHRC1 plays an important role in the activation of the AKT/ERK pathway,[30] to further elucidate molecular mechanism of *let-7c-5p* inhibiting the proliferation and migration of ESCC cells, we explored the possibility of *let-7c-5p* inhibiting AKT and EKR signaling pathways. Western blot analysis showed that transfection with *let-7c-5p* mimics in TE-1 and KYSE150 cells significantly reduced the expression of p-AKT and p-ERK, while expression of total AKT and ERK did not significantly change (Figure 7A and B), suggesting that AKT and ERK signaling pathways were inhibited. Conversely, transfection with *let-7c-5p* inhibitor in TE-1 and KYSE150 cells markedly increased expression of p-AKT and p-ERK (Figure 7C and D), suggesting that AKT and ERK signaling pathways were promoted. Collectively, these data indicate that upregulation of *let-7c-5p* inhibited AKT/ERK signaling, while downregulation of *let-7c-5p* activated AKT/ERK signaling.

CTHRC1 Reversed Let-7c-5p-Mediated AKT and ERK Signaling Pathways

First, we analyzed the knock-out efficiency of siCTHRC1 by qRT-PCR and Western blot. After transfection with

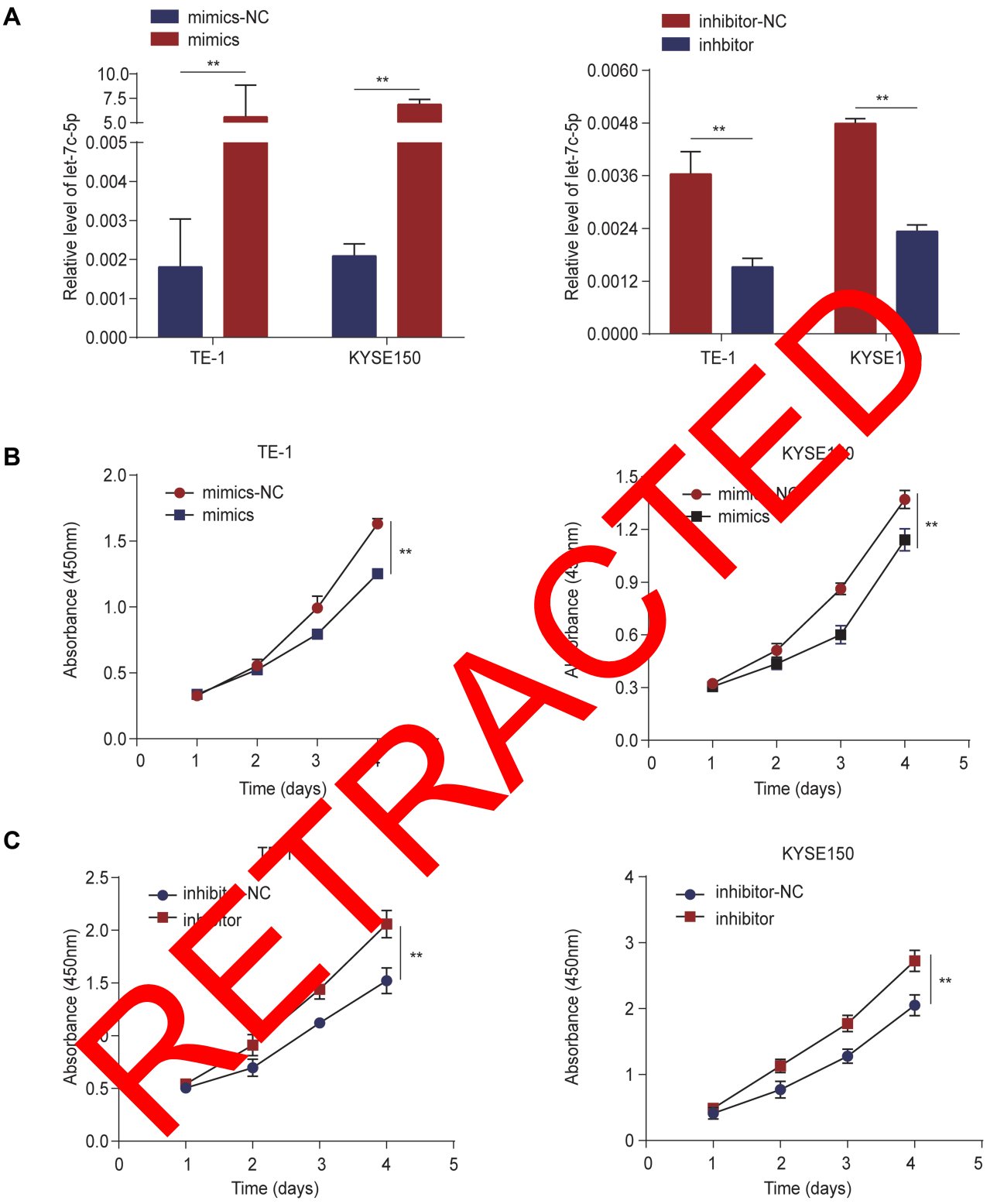

Figure 2 *Let-7c-5p* suppresses cell proliferation in ESCC. (**A**) mRNA levels of *let-7c-5p* were measured by qRT-PCR after transfection with *let-7c-5p* mimics or inhibitor in TE-1 and KYSE150 cells. (**B**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* mimics and cell viability was detected by CCK-8 array. (**C**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* inhibitor and cell viability was detected by CCK-8 array. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test (two-group comparison) or one-way analysis of variance (more than two groups). **p < 0.01.

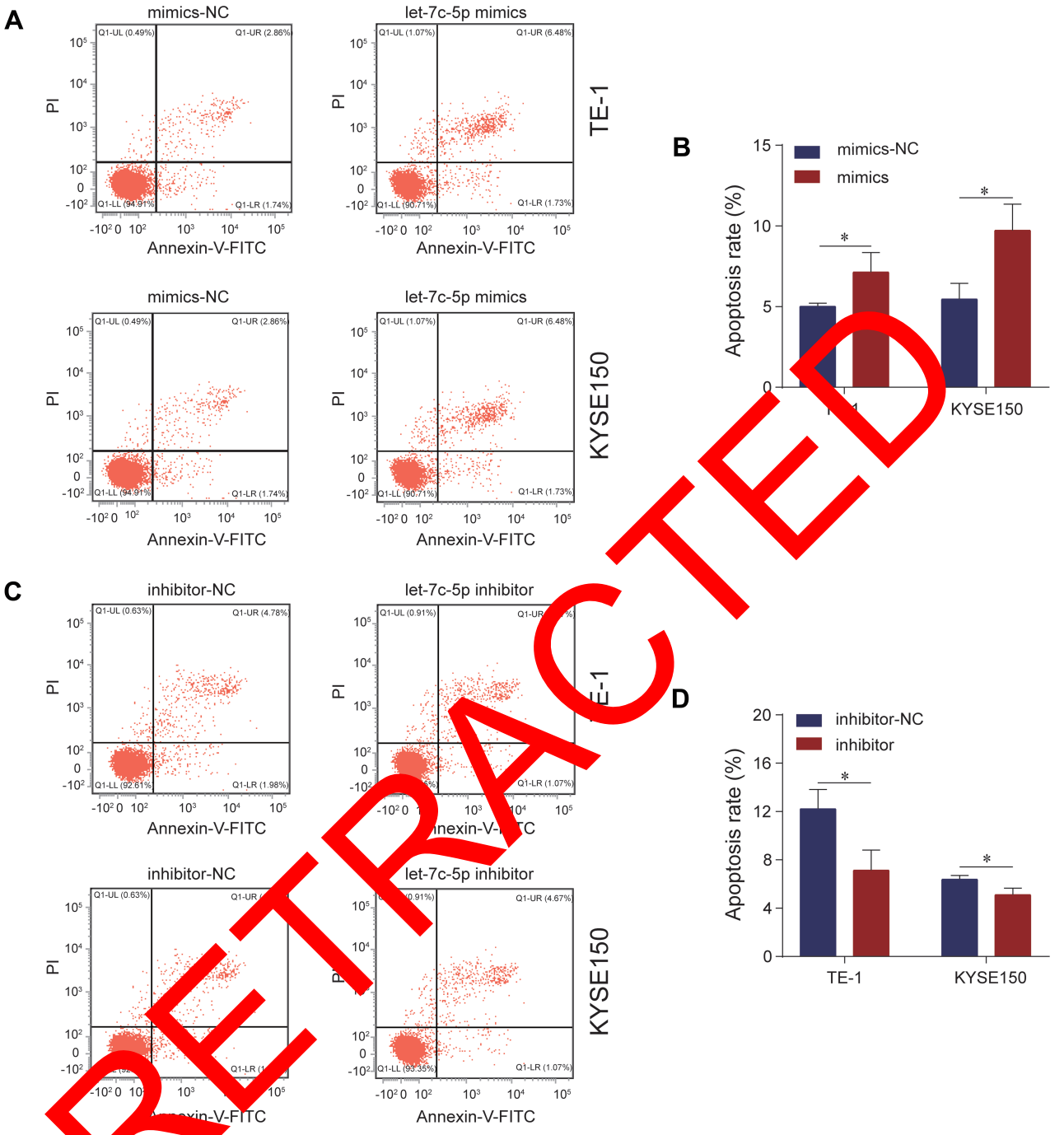

Figure 3 *Let-7c-5p* induces apoptosis in ESCC. (**A**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* mimics and the apoptotic cell percentages were measured by Annexin V-FITC/PI staining assay. (**B**) The apoptosis ratio was quantified. (**C**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* inhibitor and the apoptotic cell percentages were measured by Annexin V-FITC/PI staining assay. (**D**) The apoptosis ratio was quantified. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. *p < 0.05.

siCTHRC1-2 in TE-1 and KYSE150 cells, we found that expression of CTHRC1 was most significantly downregulated by qRT-PCR (Figure 8A and B), which was consistent with protein expression levels by Western blot (Figure 8C and D). These results revealed that among the three different small interfering RNA sequences designed, siCTHRC1-2 exhibited the best knock-out efficiency at the transcriptional and translational levels. Next, we confirmed that transfection with si-CTHRC1-2 in TE-1 and KYSE150 cells significantly reduced levels of p-AKT and p-ERK, while expression of total AKT and ERK was not markedly changed, suggesting that AKT and ERK

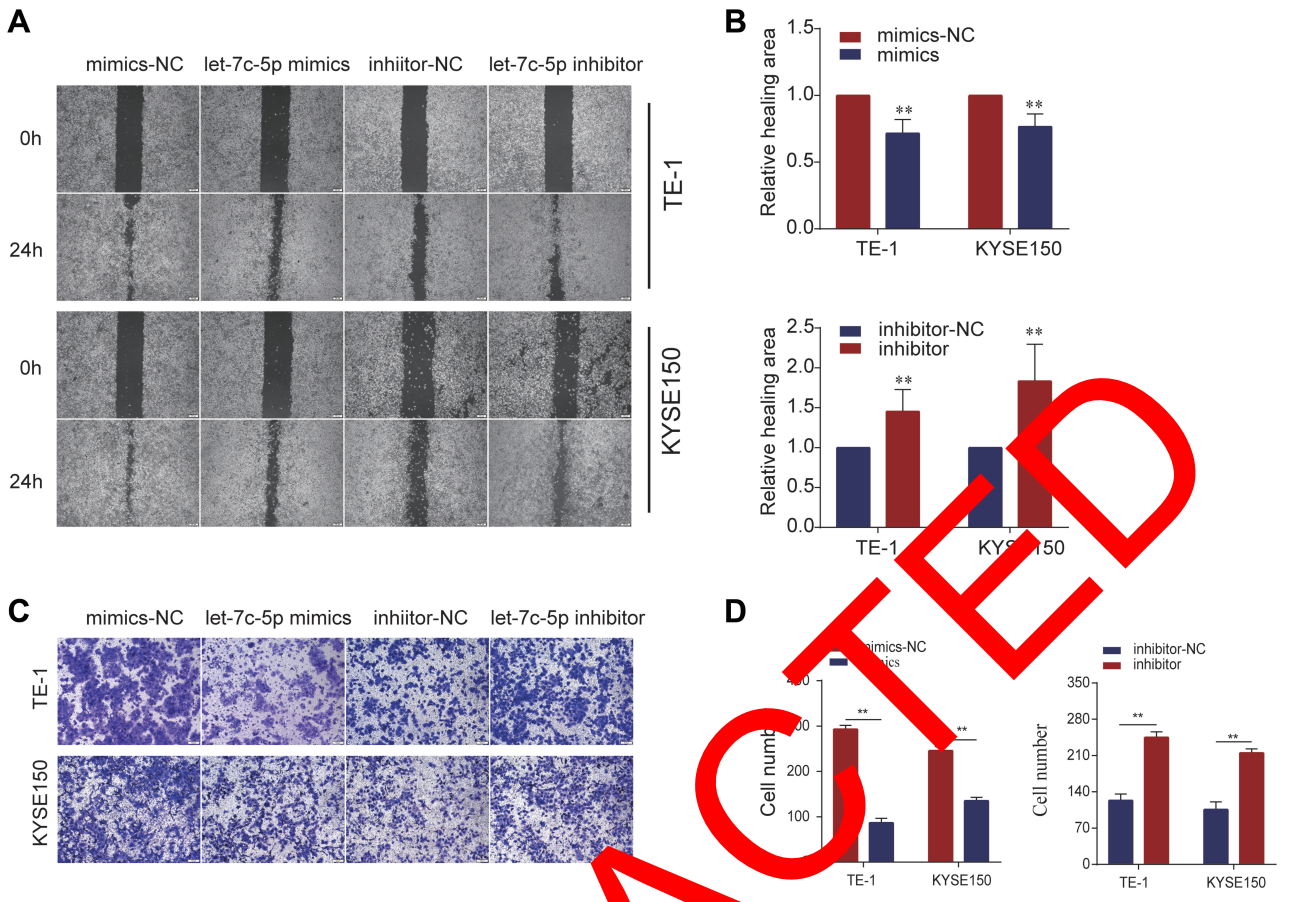

Figure 4 *Let-7c-5p* suppresses cell migration in ESCC. (**A**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* mimics or inhibitor and cell migration was assessed by wound healing assay (scale bar, 100 μm). (**B**) Wound areas were quantified. (**C**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* mimics or inhibitor and cell migration was examined by Transwell assay (scale bar, 200 μm). (**D**) Migrating cells were quantified. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. **p < 0.01.

signaling pathways were inhibited (Figure 8E and F). These results revealed that knockdown of CTHRC1 suppresses AKT and ERK signaling pathways in ESCC. In addition, after cotransfection with siCTHRC1-2 and *let-7c-5p* inhibitor, we detected protein levels of p-AKT and p-ERK in TE-1 and KYSE150 cells. Results showed that inhibiting CTHRC1 inhibited expression of p-AKT and p-ERK activated by *let-7c-5p* inhibitor (Figure 8G and H). In summary, these results indicate that *let-7c-5p* suppresses AKT and ERK signaling pathway with the inactivation of CTHRC1, consequently inhibiting cell proliferation and migration.

Let-7c-5p Inhibites ESCC Progression in vivo

We detected tumor suppressor effect of *let-7c-5p* in BALB/c-nu mice using agomir upregulating *let-7c-5p* and antagomir downregulating *let-7c-5p*. After 3 weeks of observation, the injection of agomir showed a tumor growth inhibitory effect, while antagomir showed an effective tumor growth promotion effect (Figure 9A and B). Protein expression levels of CTHRC1 in tumor tissues were evaluated by Western blot. Compared to agomir-NC, protein levels of CTHRC1 in agomir group were significantly reduced. Compared to antagomir-NC, protein levels of CTHRC1 in antagomir group were significantly increased (Figure 9C and D). These results indicate that *let-7c-5p* overexpression inhibits tumor growth, while *let-7c-5p* inhibition promotes tumor growth in vivo.

Discussion

In this study, we observed that *let-7c-5p* is a tumor suppressor in ESCC and that overexpression of *let-7c-5p* inhibited proliferation and migration and promoted apoptosis by negatively regulating CTHRC1 and indirectly regulating the AKT and ERK signaling pathways. These

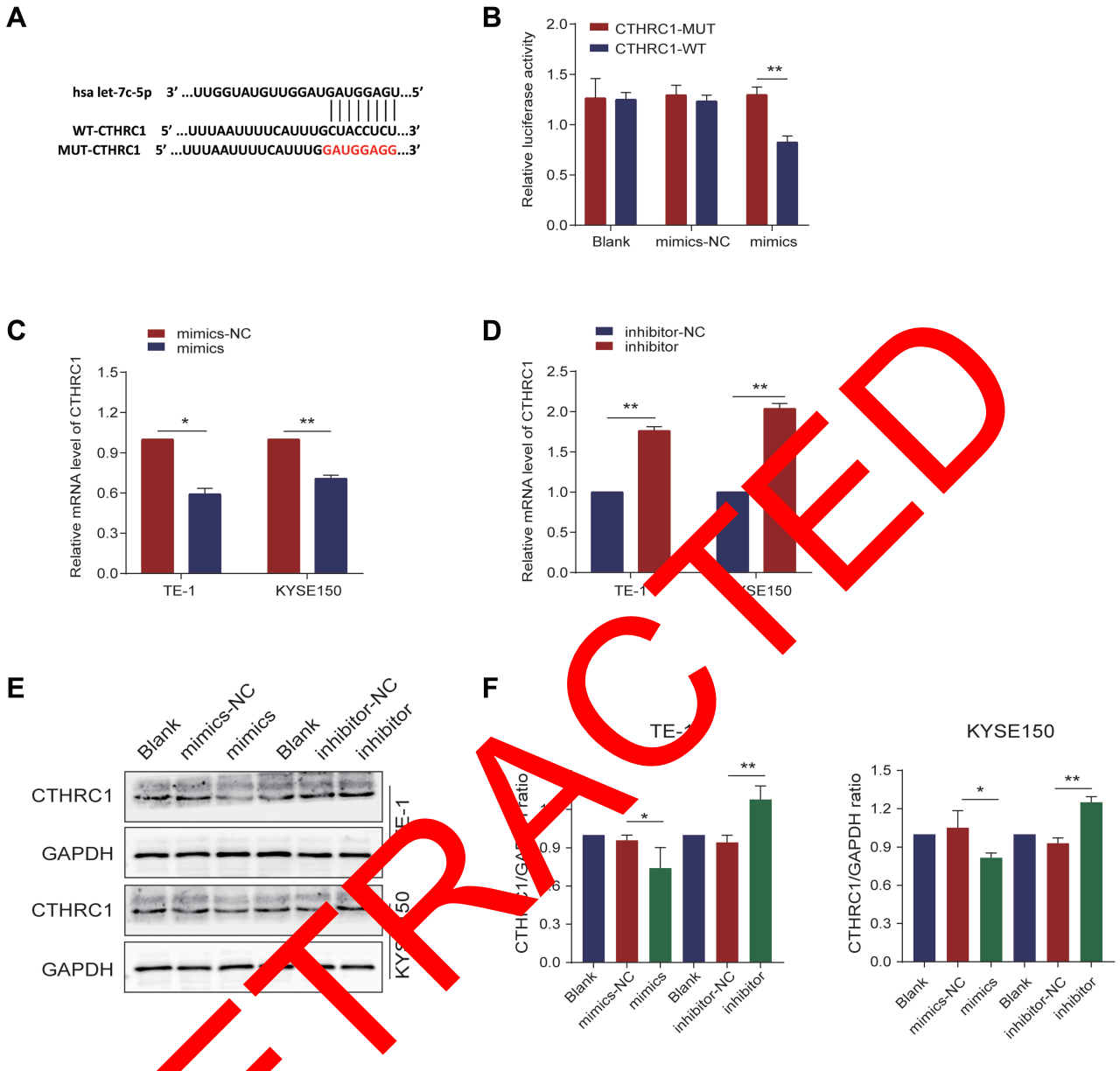

Figure 5 CTHRC1 is a di... target ...*let-7c-5p*. (**A**) WT and Mut sequences of the putative *let-7c-5p* target sequences of CTHRC1 3'-UTR. (**B**) Dual-luciferase activity assay showing the effect of *let-7c-5p* ... CTHRC1 3'-... luciferase activity in HEK-293T cells. (**C**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* mimics and mRNA levels of CTHRC... assessed ... qRT-PCR. (**D**) TE-1 and KYSE150 cells were transfected with *let-7c-5p* inhibitor and mRNA levels of CTHRC1 were examined by qRT-PCR. (**E**) TE-... and KYS...50 cells ... transfected with *let-7c-5p* mimics or inhibitor and protein levels of CTHRC1 were analyzed by Western blot. (**F**) The CTHRC1/GAPDH r... was quant...ed. All result...e shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. *p < 0.05 and **p <...

findings indicat... that *let-7c-5p* represents a possible therapeutic strategy for ESCC. It has been reported in a variety of solid tumors that *let-7c-5p* acts as a tumor suppressor, inhibiting cell proliferation, migration and invasion.[18–23] Although cluster analysis has found that *let-7c* is downregulated in ESCC tissues, the specific role and underlying mechanisms of *let-7c* in ESCC have not been further elucidated. To explore the mechanism of *let-7c-5p* in ESCC, first, we confirmed that *let-7c-5p* was clearly downregulated in ESCC tissues and five ESCC cell lines (TE-1, TE10, TE11, KYSE140 and KYSE150) compared to normal tissues and cells, which was consistent with the previously reported cluster analysis. Then, we analyzed the relationship between *let-7c-5p* and clinicopathological parameters, demonstrating that reduced expression of *let-7c-5p* was correlated with advanced TNM staging and lymph node metastasis. Moreover, we found that *let-7c-5p* has high sensitivity and specificity in both early and

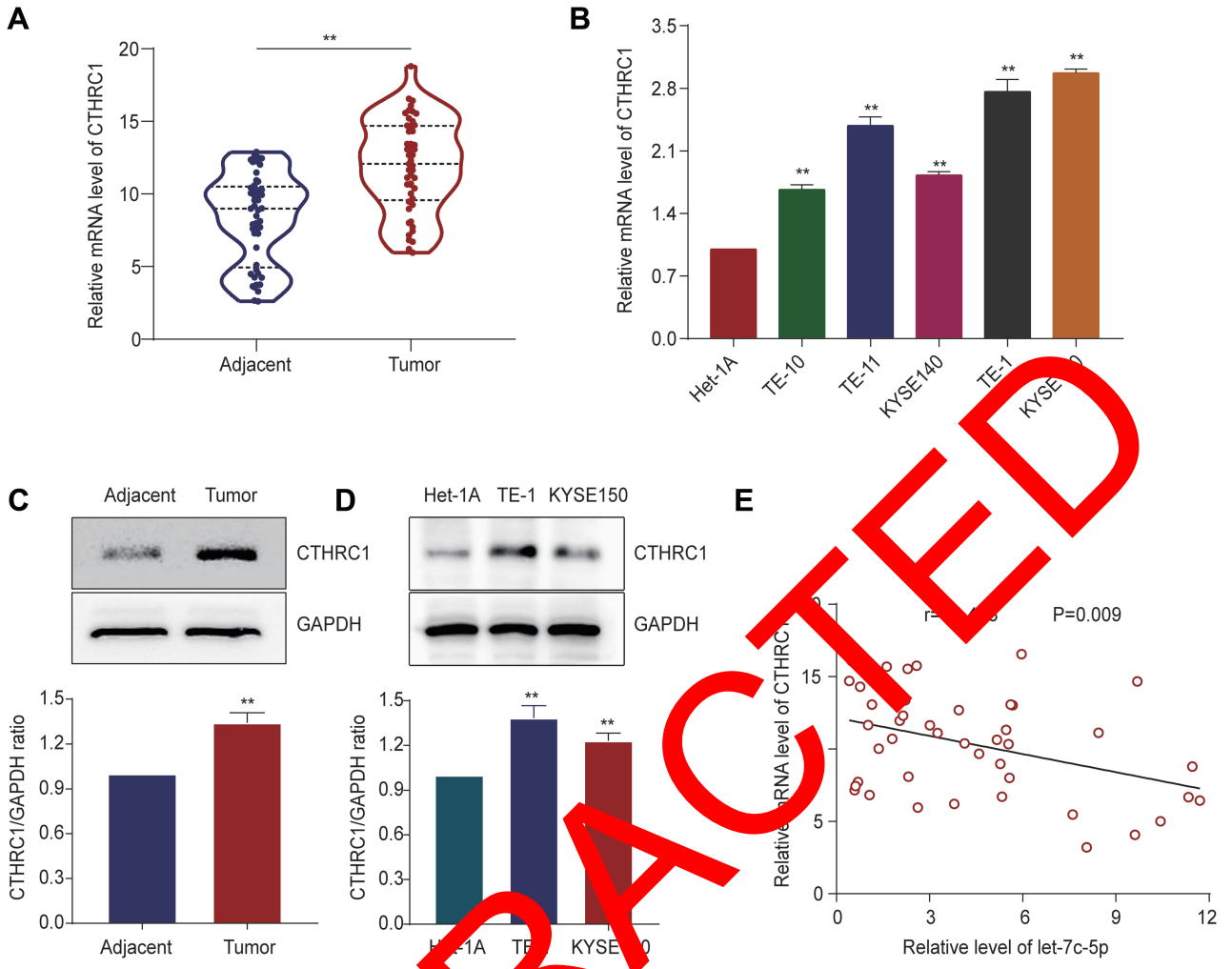

Figure 6 Expression of CTHRC1 in ESCC and its correlation with *let-7c-5p*. (**A**) Higher mRNA expression of CTHRC1 in 50 pairs of ESCC tissues compared to adjacent normal tissues by qRT-PCR. (**B**) Higher mRNA expression of CTHRC1 in five ESCC cell lines (TE-1, TE-10, TE-11, KYSE140 and KYSE150) compared to Het-1A by qRT-PCR. (**C**) Higher protein expression of CTHRC1 in ESCC tissues compared to adjacent noncancerous tissues by Western blot. (**D**) Higher protein expression of CTHRC1 in TE-1 and KYSE150 cells compared to Het-1A by Western blot. (**E**) Negative correlation between CTHRC1 and *let-7c-5p* in ESCC tissues. Statistical analysis was performed with Pearson's correlation analysis ($r = -0.418$, $P = 0.009$). All results are shown as the mean ± SD of three separate experiments (n=3) **p < 0.01.

late ESCC by ROC curve analysis, suggesting that *let-7c-5p* represents as a potential diagnostic biomarker in ESCC. These results revealed that *let-7c-5p* might have significant molecular biological functions in ESCC. Interestingly, we found that overexpression of *let-7c-5p* significantly inhibited proliferation, migration, and promoted apoptosis in vitro by functional experiments. We further found that *let-7c-5p* overexpression inhibited tumor growth, while *let-7c-5p* inhibition promoted tumor growth in tumor xenograft models. Our findings indicate that *let-7c-5p* might be a useful treatment strategy for ESCC.

Many studies have reported that CTHRC1 is an oncogenic factor that promotes tumor progression and metastasis. Mounting evidence has found that some miRNAs and CTHRC1 have a targeted regulatory relationship. For example, Lai YH et al confirmed that *miR-30c* inhibits proliferation, migration and invasion of breast cancer cells by targeting CTHRC1.[33] Furthermore, Chen G et al found that *miR-155-5p* regulates malignant biological behavior of hepatocellular carcinoma by negatively regulating CTHRC1 and affecting the Wnt/β-catenin signaling pathways.[34] However, the relationship between *let-7c-5p* and CTHRC1 remains unclear. In this study, we found that CTHRC1 is significantly upregulated in both ESCC tissues and cell lines. Further analysis of mRNA expression levels of *let-7c-5p* and CTHRC1 in ESCC tissues revealed a negative correlation between these two factors. Overexpression of *let-7c-5p* significantly reduced the expression of CTHRC1, while inhibition of *let-7c-5p* restored the expression of CTHRC1. Bioinformatics

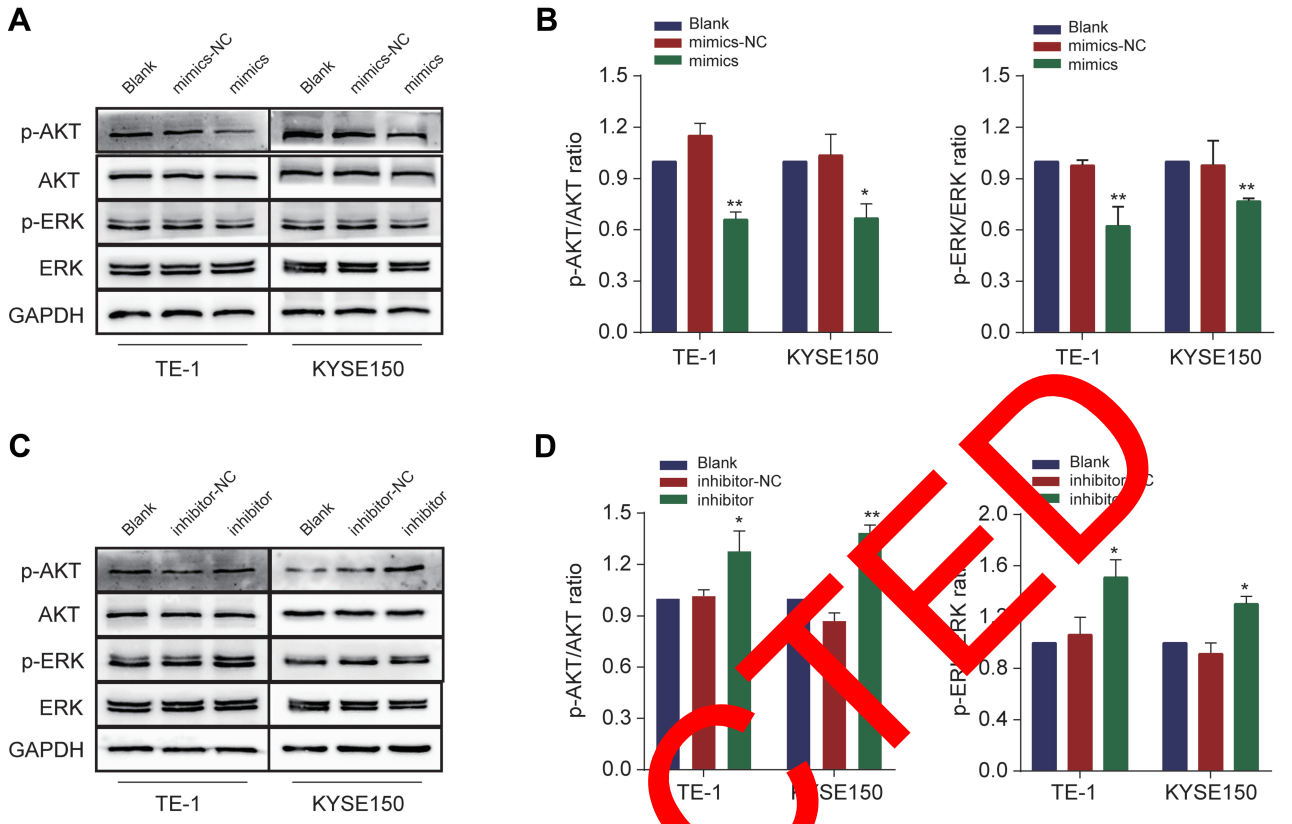

Figure 7 *Let-7c-5p* inhibits AKT and ERK signaling pathways. (**A**) Protein l[illegible] AKT, p-AKT, [illegible]-ERK were measured after transfection with *let-7c-5p* mimics in TE-1 and KYSE150 cells by Western blot. (**B**) The p-AKT/AKT and p-ERK/ER[illegible]atio [illegible]uantified. (**C**) Protein levels of AKT, p-AKT, ERK and p-ERK were measured after transfection with *let-7c-5p* inhibitor in TE-1 and KYSE150 cells by Western [illegible]t. (**D**) [illegible]T/AKT and p-ERK/ERK ratio were quantified. All results are shown as the mean ± SD of three separate experiments. Statistical analysis was performed [illegible]tudent's *t*-test. *p < 0.05 and **p < 0.01.

prediction analysis found that the sp[illegible]fic bin[illegible]g site o[illegible] *let-7c-5p* existed in the 3′UTR [illegible] CTHRC1. Next, we confirmed that CTHRC1 was [illegible]direct targe[illegible]ene of *let-7c-5p* by dual-luciferase [illegible]port a[illegible]y. In this study, we revealed for the first [illegible]me that *let-7c-5p* exerts a tumor suppressor effect [illegible]y direc[illegible]y regulating expression of CTHRC1 in ESCC.

Recently, [illegible] XX [illegible]al inno[illegible]atively studied a specific monoclo[illegible]al antib[illegible]dy agai[illegible]THRC1, which significantly reduced [illegible]ound[illegible]nd cell invasion ability in vitro in cervical c[illegible]er.[35] Many studies have reported that CTHRC1 pr[illegible]otes epithelial-mesenchymal transition (EMT), invasion, migration, or induce abnormal angiogenesis through multiple signaling pathways, thereby enhancing tumor cell invasion and metastasis.[30,32,36–38] We believe that targeted regulation of CTHRC1 and its downstream signaling pathways might represent a potential treatment for ESCC. Wang CN et al found that PI3K-Akt and MAPK pathways were the two pathways most affected by CTHRC1 knockdown through KEGG pathway analysis.[30]

In this study, we further confirmed by Western blot analysis that protein expression of p-AKT and p-ERK in TE-1 and KYSE150 cells were significantly reduced after knock down of CTHRC1, which was consistent with existing research reports. To further explore the mechanism by which *let-7c-5p* inhibited cell proliferation and migration of ESCC, our study found that overexpression of *let-7c-5p* inhibited the protein expression of CTHRC1, thereby reducing phosphorylation levels of AKT and ERK, revealing that *let −7c-5p* inhibits AKT and ERK signaling pathways by directly downregulating CTHRC1.

In the current study, we showed for the first time the vital role and potential molecular mechanism of *let-7c-5p* in ESCC. However, we only examined clinical samples from 50 patients, and further studies with additional samples are needed. In summary, our research demonstrates that expression of *let-7c-5p* is reduced in ESCC tissues and cells and that *let-7c-5p* inhibits cell proliferation and migration and promotes cell apoptosis by negatively regulating CTHRC1, indirectly regulating the AKT and ERK

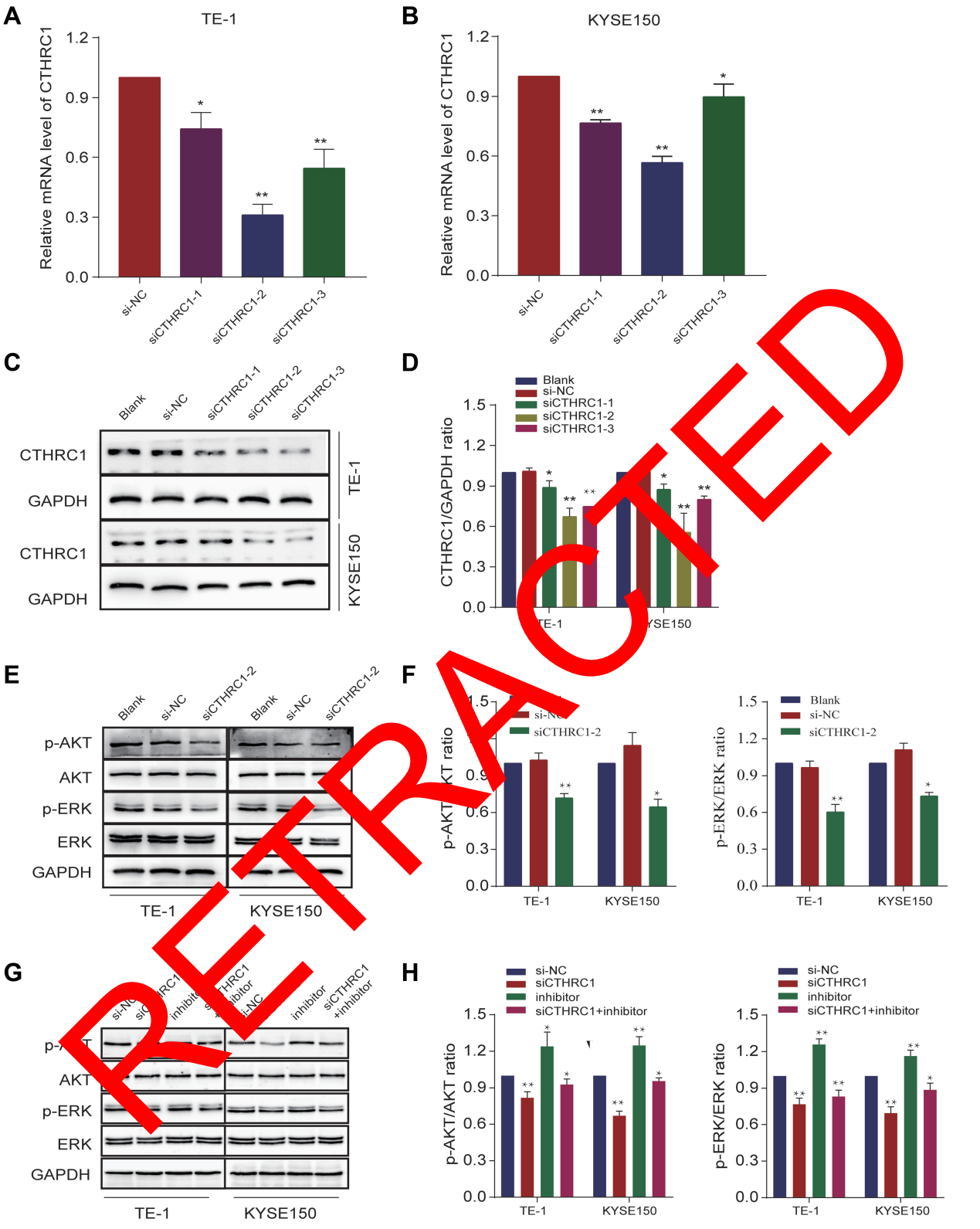

Figure 8 CTHRC1 reversed *let-7c-5p*-mediated AKT and ERK signaling pathways. (**A**) mRNA levels of CTHRC1 were measured after transfection with siCTHRC1 in TE-1 cells by qRT-PCR. (**B**) mRNA levels of CTHRC1 were examined after transfection with siCTHRC1 in KYSE150 cells by qRT-PCR. (**C**) Protein levels of CTHRC1 were detected after transfection with siCTHRC1 in TE-1 and KYSE150 cells by Western blot. (**D**) The CTHRC1/GAPDH ratio was quantified. (**E**) Protein levels of AKT, p-AKT, ERK and p-ERK were measured after transfection with siCTHRC1-2 in TE-1 and KYSE150 cells by Western blot. (**F**) The p-AKT/AKT and p-ERK/ERK ratio were quantified. (**G**) Protein levels of AKT, p-AKT, ERK and p-ERK were measured after cotransfection with siCTHRC1-2 and *let-7c-5p* inhibitor in TE-1 and KYSE150 cells by Western blot. (**H**) The p-AKT/AKT and p-ERK/ERK ratio were quantified. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. *p < 0.05 and **p < 0.01.

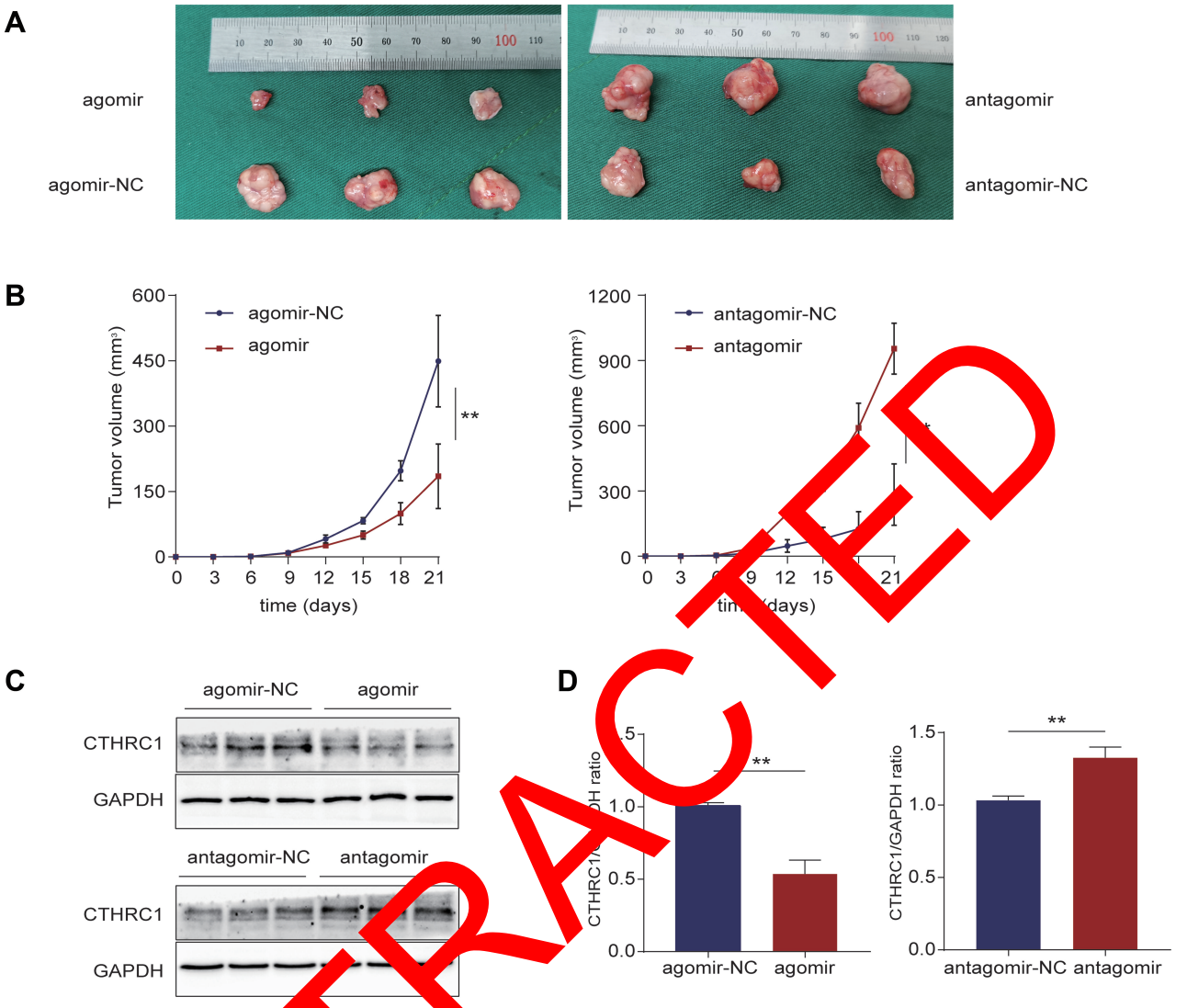

Figure 9 *Let-7c-5p* inhibits ESCC progression in vivo. (**A**) Images of nude mice tumor specimen. (**B**) The growth curves of TE-1 cells xenograft tumors after injection with *let-7c-5p* agomir and let-7c-5p antagomir. Tumor volumes are shown as the mean±SD for each group of three mice. (**C**) Protein levels of CTHRC1 in tumor tissues were detected by Western blot. (**D**) The CTHRC1/GAPDH ratio was quantified. All results are shown as the mean ± SD of three separate experiments (n=3). Statistical analysis was performed with Student's *t*-test. **p < 0.01.

signaling pathways. Future research on *let-7c-5p* may be beneficial for the diagnosis and treatment of ESCC.

Conclusion

In conclusion, this study demonstrated that *let-7c-5p* is a tumor suppressor in ESCC, and *let-7c-5p* inhibited the proliferation, migration and promoted apoptosis by directly negatively regulating CTHRC1 and indirectly regulating AKT and ERK signaling pathways. Therefore, *let-7c-5p* may represent a new molecular marker for the treatment of ESCC.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest in this work.

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