Long Noncoding RNA *LINC00518* Contributes to Proliferation and Metastasis in Lung Adenocarcinoma via the *miR-335-3p/CTHRC1* Axis

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Research

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Abstract

Background

Recently, a considerable amount of researches had been published that in some human tumours long intergenic nonprotein coding RNA 518 (LINC00518) could promote cancer proliferation and metastasis.

Methods

To directly test the effects of LINC00518 in LUAD, we treated the A549 and H1299 cells. Quantitative real-time PCR (qRT-PCR), western blot, and immunohistochemical assays were used to detect the expression of genes. CCK-8, clonogenic assays, the wound healing, migration and invasion assays to investigate the proliferation and motility of cells. Flow cytometry was used to determine the cell cycle stage. Meanwhile, a lung carcinoma xenograft mouse model was used to investigate the in vivo effects.

Results

Here we found that LINC00518 levels were up-regulated in lung adenocarcinoma (LUAD) tissues, and aberrant LINC00518 levels were particularly associated with the poor prognosis of LUAD patients. LINC00518 functions as a ceRNA to regulate the cell proliferation and tumour growth of LUAD by regulating the cell cycle and focal adhesion signalling and might be a potential synergistic therapeutic target of the FAK-inhibitor VS-6063.

Conclusions

Thus, LINC00518 could do duty for a biomarker and potential therapeutic target in LUAD.

Background

In nowadays society, lung cancer remains at the top of the list of tumour-related deaths around the world, and its incidence has increased steadily by 10% annually [1–3]. Lung cancer could be divided into two histological subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for around 85% of lung cancers, and includes adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and large cell carcinoma; SCLC accounts for 15% of lung cancers [4]. Lung adenocarcinoma (LUAD) is one of the major types of lung cancer [5]. Therefore, the mechanism of proliferation, metastasis, and drug resistance of LUAD should be explored extremely urgently.

Long noncoding RNAs (lncRNAs) are noncoding RNAs of more than 200 nucleotides, that can be used as biomarkers for the diagnosis and prognosis of numerous human tumours [6–8]. With the development of research, it has been explored that an increasing number of lncRNAs had existed as an indispensable part

in the malignant progression of lung cancer. Long intergenic non-protein coding RNA 518 (LINC00518), has been known to promote and up-regulated cancer cell proliferation and metastasis in cervical cancer, breast cancer, and malignant melanoma [9–12]. It also makes a contribution to chemotherapeutic drug resistance in prostate cancer and breast cancer [13, 14]. In addition, it could induce radioresistance by regulating glycolysis in melanoma [15]. Some researchers have shown that LINC00518 acts as an oncogene to facilitate tumour progression in NSCLC by regulating an RNA-based network[16]. Nevertheless, the accurate role and molecular mechanism of LINC00518 in LUAD are still undetermined.

Certain specific lncRNAs, substantial studies have demonstrated that, may serve on competitive endogenous RNAs (ceRNAs) in tumorigenesis and development [17, 18]. Some researches have been made to demonstrate that, in melanoma, prostate cancer, and breast cancer, LINC00518 plays a similar role [11, 13, 14]. Thus, we conjectured that LINC00518 might be the same in LUAD and constructed the ceRNA network of LINC00518.

Collagen triple helix repeat containing 1 (CTHRC1), what we know about, is a chondrocyte-specific, secreted glycoprotein that was initially discovered in a rat model of balloon-injured arteries [19–24]. It is frequently detected in several solid tumours that the expression of CTHRC1 is high, such as breast ductal carcinoma, hepatocellular carcinoma, gastric cancer, colorectal cancer, and melanoma [25–29]. It has been reported that high expression of CTHRC1 was meaningfully correlated with metastasis in patients with NSCLC and that over-expression of CTHRC1 might be involved in tumour poor prognosis and angiogenesis in LUAD [30].

As a sort of anchoring junction, focal adhesion dominantly is mediated by integrins that integrate the surrounding extracellular matrix (ECM) with the actin cytoskeleton[31]. As the kernel constituents of focal adhesion, 24 transmembrane αβ heterodimers constituted the integrin family, which generated from selective noncovalent unions between 18 α and 8 β subunits. Integrin β3, among them, plays a predominant role and is significantly associated with malignant phenotypes of tumours[32].

This study aims to identify that LINC00518 sponges miR-335-3p to activate CTHRC1 transcription and integrinβ3/FAK signalling, which are required for LUAD proliferation and metastasis. We also showed that LINC00518 is highly expressed in LUAD tissue and plays a critical role in prognosis, which could serve as a predictive biomarker and potential therapeutic target for LUAD.

Materials And Methods

Patients and tissue samples

We obtained twenty primary lung adenocarcinoma tissues and adjacent normal tissues (ANTs) from the First Affiliated Hospital of Soochow University (**Additional file 2: Table S2**). There are no patients who received radiotherapy or chemotherapy before surgery. Two pathologists diagnosed the histological characteristics of the tissue, independently. And the study was approved by the Human Research Ethics Committee of the First Affiliated Hospital of Soochow University. Moreover, we obtained informed consent

from all patients. Lung adenocarcinoma samples from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/. Accessed 2 May 2020.) were also included in this study.

Dataset Analysis

We screened the differentially expressed lncRNAs from the TCGA database with R version 3.63 (fold change=1.0, P<0.05). The survival status and survival time of the patients were downloaded from the TCGA database (https://portal.gdc.cancer.gov/. Accessed 2 May 2020.) to obtain a prognostic model. Next, we combined the information about 309 patients with complete clinical data on TCGA-LUAD with the risk score, and the independent prognostic factor was analyzed by using the “survival” package of R software.

The Cerna Network

We used the Cytoscape (v3.6.0) to construct the network of the LINC00518-miRNA-target gene to visualize their interactions. And we predicted the LINC00518/miRNA interaction by using miRcode (https://www.mircode.org/. Accessed 2 May 2020.), and identified the target genes of the miRNAs by using miRDB (https://www.mirdb.org/. Accessed 2 May 2020.), TargetScan (https://www.targetscan.org/. Accessed 2 May 2020.), and miRTarBase (https://mirtarbase.mbc.nctu.edu.tw/. Accessed 2 May 2020.).

Materials

We purchased the cells from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China, including A549, H1299 (lung adenocarcinoma), H460 (large cell carcinoma), H226 (lung squamous cell carcinoma), and BEAS-2B (human immortalized normal epithelial cells) cells. And cultured them in RPM1 1640 medium with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) at 37 ℃ in a 5% CO_2 atmosphere.

Rna Interference

MiR-335-3p mimics, miR-335-3p inhibitors, and the related negative control (NC) were purchased from GenePharma (Suzhou, China). The GenePharma (Suzhou, China) directly synthesized the small interfering RNA (siRNA) and sequences corresponding to the target sequences for us. The siRNA constructs were as follows: si-NC: 5'-UUCUCCGAACGUGUCAC GUTT-3'; si-LINC00518–1: 5'-CCGGGCTAGATGGAACCTTAGTGAACTCGAGTTCACTAA GGTTCCATCTAGCTTTTT-3'; si-LINC00518–2: 5'-CCGGCACCTCCAAAGTGACGACTTAC TCGAGTAAGTCGTCACTTTGGAGGTGTTTTT-3'; si-CTHRC1–1: 5'-CACAUUCAAUGGAG CUGAATT-3'; si-CTHRC1–2: 5'-CGGAGUGUACAUUUACAAATT-3'. Next, insert short hairpin RNA (shRNA) and its corresponding control sequences into the lentivirus vector (GenePharma, Suzhou, China). To obtain cells stably expressing miR-335-3p or LINC00518 shRNA, lung

adenocarcinoma cells were infected with lentiviruses. Then, we performed with Lipofectamine 2000 to transfect siRNA into cells according to the instructions of the manufacturer.

Quantitative Real-time Pcr Analysis

As we described previously, the detailed processes were performed[33]. And we listed the primers used in the study in **Additional file 1: Table S1**. We normalized the CT values of the gene mRNA levels to those of β-actin, the internal control. The $^{\triangle\triangle}$Ct method was applied to calculate the relative quantities of these mRNAs.

Rna Binding Protein Immunoprecipitation (Rip)

According to the manufacturer's protocol, the RIP kit (BersinBio, Guangzhou, China) was used to accomplish RIP analysis. Firstly, lyse A549 and H1299 cells were lysed by RIP lysis buffer. Secondly, we incubated the lysate products, pre-conjugated with anti-IgG or anti-Ago2 antibody, with magnetic beads at 4°C for 12–16 hours or overnight. Then, to obtain purified RNA, we used protease K to eliminate proteins. Last, the expression level of LINC00518 and miR-335-3p were determined by qRT–PCR. We performed each experiment in triplicate.

Cell Proliferation Analysis

Using Cell Counting Kit-8 (Dojindo, Shanghai, China) to examine cell proliferation. First, we cultivated LUAD cells into 96-well plates (3000 cells per well) treated with a short interfering RNA (siRNA) or plasmid and for 24, 48, and 72 h further grown under normal culture conditions. According to the manufacturer's instructions, we determined cell viability. Moreover, we used the clonogenic assay to detect cell proliferation. In brief, we diluted cells transfected with si-LINC00518, si-CTHRC1, si-NC, miR-335-3p mimics, or miR-NC in the complete culture medium, and reseeded 200 cells into a 60-mm plate. Depending on the cell growth rate, after about 7–10 days incubating, foci formed by at least 50 cells were stained with Giemsa and counted.

Transwell Assays

To fulfill cell migration and invasion assays, transwell inserts in 8.0 μm in size (Corning, New York, NY, USA) were used. For the migration assay, firstly, we added 800 μl RPMI 1640 medium with 10 % FBS to each lower chamber of the insert. Secondly, trypsinize the stable cells, and then we seeded 4×10^4 cells with medium containing 1% FBS into the upper chamber and incubated them at 37 ℃ for 24 h. Furthermore, fix the cells migrated onto the lower surface of the insert with 100% methanol for 20 min, air-dried for 10 min, stained with 0.1% crystal violet overnight and washed with 1×PBS twice. Last, photograph and count the cells. For the invasion assay, coat the inserts with Matrigel matrix (BD Science,

Sparks, MD, USA) diluted in serum-free medium and then incubate them at 37 ℃ for 2h. The following steps were the same as the migration assay. We performed each experiment in triplicate.

Wound Healing Assay

We seeded the stable cells of A549/H1299 into 6-well tissue culture plates at a density of ~ 70–80 % confluence as a monolayer. And then scratch the monolayer. We used a fresh 10-μl pipette tip, gently and slowly, to scratch across the centre of the well, so as to a resulting gap distance equivalent to the outer diameter of the end of the tip. Next, make another scratch perpendicular to the first to create a cross. Two gentle washes with 1 × PBS should be done to remove the detached cells. Fresh medium was replenished within the wells, and cells were cultured for an additional 24 h. A microscope (CKX41, Olympus) was used to observe and image the cells at the same magnification and settings.

Cell Cycle Analysis

We purchased the cell cycle analysis kit from Beyotime Biotechnology (Shanghai, China). After suspending in 80% ethanol at 4 ℃ overnight, the cells were stained with a PI/RNase mixture, particularly in the dark, at 37 ℃ for 30 min to the cell cycle analysis. Then, we detected the stained cells in a FACSCalibur system (Beckman Coulter, Brea, CA, USA).

Western Blot Analysis

We lysed the transfected cells and tissues by using 1× RIPA buffer with phosphatase inhibitor and protease inhibitor (Apexbio). Afterward put them on ice for 30 min to shake, and then centrifuge them at 4°C and 12,000 g for 15 min. Next, we used the 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) to separate the extracted proteins and then transfer them to NC membranes (Millipore, Billerica, MA, USA). The following antibodies were used in the study: anti-CTHRC1 (ab85739), anti-CyclinD1 (ab226977), anti-Integrin-αV (ab179475) (Abcam, London, UK), anti-MMP-9 (13677), anti-Fak (13009), anti-p-Fak (Ser397) (8556) (Cell Signaling Technology), and anti-Integrin β3 (A19073) (Abclonal, Wuhan, China). Anti-β-actin (CW0096M), anti-rabbit (CW0103), and anti-mouse (CW0102) secondary antibodies were purchased from Cowin.

Luciferase Reporter Assay

We inserted the fragments of the CTHRC1 3’-UTR and LINC00518 containing the binding site of miR-335-3p into the pMIR-REPORT vector. Then, we cotransfected with luciferase reporter plasmids and related oligonucleotides in A549 and H1299 cells. Plasmids with mutation-binding sites were used as controls. To detect the luciferase activity of reporter plasmids, we used the Dual Luciferase Reporter Assay System purchased from the Promega [12].

Co-immunoprecipitation (Co-ip) Assay

Lysed the cell for 30 min with 1 ml of RIPA buffer purchased from the Cell Signaling Technology (Danvers, MA, USA). Next, scraping to collect the cells and the protein in the lysates was incubated for more than 12 h with anti-CTHRC1 (Abcam, London, UK) or normal rabbit IgG antibody at 4 ℃ with rotation. Overnight, incubate the mixture with protein A/G beads or anti-c-Myc magnetic beads at 4 ℃ for 4 h. Boil the beads in the 2×SDS protein loading buffer, after washing with lysis buffer triples, and then subject them to Western blot analysis.

Animal Experiments

We obtained the female BALB/c nude mice (about 3–4 weeks old) from the Experimental Animal Center of Soochow University and which bred under pathogen-free conditions. We carried out all animal experiments in accordance with the Guide for the Care and Use of Experimental Animals Center of Soochow University. Aim to find the xenograft model of lung carcinoma, we subcutaneously inoculated a total of 3.0×10^6 A549 cells into the flanks, and the female mice were randomly divided into three groups (five mice per group): LV-NC, sh-LINC00518–1, sh-LINC00518–2. Finally, determine the tumour volume (V) by using the Vernier caliper to measure the width (W) and length (L) and apply the following formula: $V = (L \times W^2) \times 0.5$.

Immunohistochemistry (Ihc)

Fixed all specimens with formalin and embedded in paraffin. It was deparaffinized with xylene after the specimen was sectioned and then hydrated with alcohol. Then incubate the sections of specimens with primary antibodies against CTHRC1 (Abcam, London, UK) and integrin β3 (Abclonal, Wuhan, China) at 4°C overnight. Next, we incubated the sections of specimens with the corresponding biotinylated secondary antibodies. As we described before, the reactions were developed using the DAB kit (BD Bioscience, San Jose, CA, USA), and the sections were counterstained with hematoxylin [34].

Membranous PSMA quantification for each sample was determined by a pathologist blinded to clinical and molecular data using modified H-Score(H-SCORE=$\sum$pi×i)=percentage of weak intensity area ×1)+(percentage of moderate intensity area ×2)+(percentage of strong intensity area ×3), to determine the overall percentage of mPSMA positivity across the entire stained tumour sample, yielding a range from 0 to 300 [35, 36].

Statistical analysis

Use the paired Student's t-test to assess the differences between lung adenocarcinoma tissues and adjacent normal tissues for LINC00518 analysis. Use the unpaired Student's t-test to assess the differences between the two groups. All results were presented as the means ±SD. We performed the

statistical analyses using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Differences for which P<0.05 were considered significant.

Results

LINC00518 is upregulated in lung adenocarcinoma (LUAD) tissues and plays a significant role as a prognostic factor

To screen potential oncogenic lncRNAs in LUAD, we compared lncRNA expression in LUAD and normal tissues, which were downloaded from the TCGA database. And the volcano map showed all differentially expressed lncRNAs in LUAD from TCGA (Fig. 1a). Then we used COX and LASSO regression to analyze all differentially expressed lncRNAs. The lambda model of the LASSO regression is shown in Fig. 1b. We chose the minimum lambda, and 39 lncRNAs were selected (Fig. 1c **and Additional file 3: Table S3)**. Next, we detected the expression of 39 lncRNAs and detected that the expression of LINC00518 in lung adenocarcinoma tissues was higher than that in normal from TCGA dataset by using the GEPIA (http://gepia.cancer-pku.cn/) (Fig. 1e). To verify this conclusion, we analyzed the LINC00518 levels in 20 LUAD tissues and para-carcinoma tissue samples and found the same results (Fig. 1f). We analyzed the overall survival (OS), disease-free survival (DFS), disease-specific survival (DSS), and progression-free survival (PFS) of 483 LUAD patients with high or low LINC00518 expression. The results indicated that LINC00518 played an indispensable role in the prognosis of LUAD patients. (Fig. 1d)

The Cerna For Linc00518

LINC00518 takes a place in the proliferation of some human tumours through a ceRNA mechanism. We supposed that LINC00518 has a similar effect in lung adenocarcinoma, and used the Cytoscape (v3.6.0) to construct a network about LINC00518-miRNA-target genes to provide the visual interrelationships. We predicted the LINC00518/miRNA interaction by using miRcode, and we identified the target genes of the miRNAs via miRTarBase, TargetScan, and miRDB. (Fig. 1g) In the network above, miR-335-3p caught our eyes since the expression of miR-335-5p, which are the different arms of mir-335, was down-regulated in LUAD[37]. CTHRC1, the predicted target of miR-335-3p, was increased in LUAD[38].

LINC00518 knockdown suppresses LUAD cell proliferation, colony formation, migration and invasion, and arrests the cell cycle

Lung adenocarcinoma cells (A549 and H1299) expressed higher LINC00518 levels than human bronchial epithelial cells (BEAS-2B) (Fig. 2a). Several attempts had been made to explore the biological effect of LINC00518 in LUAD (A549/H1299) cells. First of all, knock down the expression of LINC00518 by the LINC00518-targeting small interfering RNA (siRNA) in A549 and H1299 lung adenocarcinoma cells. After establishing siRNA efficacy, phenotypic and functional analyses were conducted in A549 and H1299 cell lines to determine the functions of knocking down the expression of LINC00518. The Cell Counting Kit-8 assay identified that the proliferation was expressively decreased in LINC00518-reduced cells compared

to that in control (Fig. 2b). Additionally, the cell survival capability of low-expression of LINC00518 was decreased by colony formation assays (Fig. 2c). Next, we had access to the wound-healing and Transwell assays to measure the impression of LINC00518 on the migration and invasion of LUAD cells, respectively. Lower migratory and invasive capabilities had been represented in LINC00518 siRNA-transfected LUAD cells than in the controls (Fig. 2d, e). To determine how LINC00518 knockdown suppressed cell proliferation in LUAD cells, we conducted KEGG pathway analysis using the TCGA database by Gene Set Enrichment Analysis (GSEA) 4.1.0 software. The results showed that LINC00518 may be essential to the cell cycle and focal adhesion (Fig. 2f). Then, we detected that the proportion of cells in the S phase was efficiently lower and in the G0/G1 phase was efficiently higher in LUAD cells by examining the distribution of cell cycle phases via the flow cytometry (Fig. 2g). Our results suggest that LINC00518 could promote cell migration and invasion in lung adenocarcinoma.

MiR-335-3p is directly targeted by LINC00518 and suppresses the LUAD cell proliferation, colony formation, migration, and invasion

First, in A549 and H1299 cells, we detected the expression of LINC00518 transfected with miR-335-3p and detected the expression in the contrast cells which were transfected with miR-NC. The level of LINC00518, consistent with the expression of miR-335-3p, was down-regulated in cells transfected with the miR-335-3p mimics, as detected by using qRT–PCR (Fig. 3a). To make sure of this prediction, we next constructed LINC00518 luciferase plasmids containing the wild-type and mutant miR-335-3p binding sites. And then, as shown in Fig. 3b, the results detected that the luciferase activity was inhibited by miR-335-3p in cells that were transfected with the wild-type LINC00518 3’-UTR, however, was not repressed in cells containing the mutant construct. Moreover, the RIP assay in A549/H1299 cells further verified the direct interaction between LINC00518 and miR-335-3p. The relative RNA expression levels of LINC00518 and miR-335-3p, compared with IgG antibody, were notably increased in the immunoprecipitation formed by the Ago2 antibody (Fig. 3c).

We induced overexpression of miR-335-3p in LUAD cells by using mimics, to identify the function of miR-335-3p, and studied their impressions on cell growth. LUAD cells over-expressing miR-335-3p had expressively lower ability to proliferation than the control cells was shown by CCK-8 assays (Fig. 3d). The results were confirmed by the colony formation assays, which indicated that miR-335-3p could inhibit LUAD cells proliferation (Fig. 3f). Transwell assays of LUAD cells further forecasted that over-expression of miR-335-3p relatively suppressed the migratory ability of NSCLC cells (Fig. 3e). To determine the effects of miR-335-3p transfection in A549/H1299 cells, the wound-healing assay was performed. It is shown in Fig. 3g that, in the cells transfected with the miR-335-3p mimics, the speed at which cells migrated towards the scratch was slower than in the control. In summary, these results demonstrate that miR-335-5p might act as the tumour suppressor in LUAD.

CTHRC1 is regulated by miR-335-3p and increases the proliferation, migratory and invasive abilities of LUAD cells

The expression of CTHRC1, in A549/H1299 cells, could be detected transfected with miR-335-3p mimics, and at the same time be detected in the control cells which were transfected with miR-NC. The level of CTHRC1, corresponding with miR-335-3p, was down-regulated in cells transfected with the miR-335-3p mimics, as determined via qRT–PCR (Fig. 3h). To determine this prediction, we constructed the CTHRC1 wild-type 3'-UTR and CTHRC1 MUT 3'-UTR and performed the dual-luciferase reporter assay in A549 and H1299 cells. It is significantly validated that the luciferase activity was inhibited by miR-335-3p in cells transfected with the wild-type CTHRC1 3'-UTR, however, was not repressed in cells containing the mutant construct (Fig. 3i).

We established the CTHRC1-targeting small interfering RNA (siRNA) in A549 and H1299 cells. The growth of the control cells, determined by CCK-8 and colony formation assays, was observably promoted compared with that of the cells with low CTHRC1 expression (Fig. 4a,b). Moreover, knockdown of CTHRC1 suppressed the migratory and invasive abilities of A549 and H1299 cells were shown by wound healing and transwell assays (Fig. 4c,d). The flow cytometry results also showed that CTHRC1 can promote proliferation by affecting the cell cycle in LUAD cells (Fig. 4e).

CTHRC1 could affect integrin β3/FAK signalling and LINC00518 might be a potential synergistic therapeutic target of the FAK-inhibitor VS-6063

First, the expression data of mRNA in LUAD was downloaded from the TCGA database, and we implemented an analysis of co-expression between CTHRC1 and integrin (Fig. 5a). Then, put the information into the DAVID database (https://david.ncifcrf.gov/) for functional analysis, including the data from TCGA database, the analysis of co-expression performed before with CTHRC1 coefficient of 0.3, $P < 0.05$, and the selected genes. It could be seen that CTHRC1 could be clustered in cell cycle and focal adhesion pathways (Fig. 5b). To determine whether CTHRC1 could affect the integrin β3/FAK signalling or not, we used Western blotting analysis (Fig. 5c). It could be revealed by co-immunoprecipitation of integrin β3 and CTHRC1 that endogenous integrin β3 of LUAD cells was immunoprecipitated by CTHRC1 antibody (Fig. 5d). Then we used the H-Score to judge the expression of CTHRC1 and integrin β3. The results of IHC staining showed a correlation of expression between them in tissues (Fig. 5e, **Additional file 4: Table S4 and Additional file 6: Fig. S2**).

To further explore the clinical significance of the study, we examined the IC50 of VS-6063, the FAK inhibitor, in A549 and H1299 cells, and the results indicated that the miR-335-3p inhibitor could elevate the IC50 of VS-6063 (Fig. 5g). CCK-8 assays also showed that the cells with VS-6063(5 μmol/L) had significantly lower proliferation ability (Fig. 5f, h). In addition, we used Transwell assays to show that VS-6063 could suppress the migratory and invasive abilities of LUAD cells when miR-335-3p was inhibited (Fig. 5i **and Additional file 5: Fig. S1a)**. FAK signalling, which is the drug target of VS-6063, was induced by Western blotting analysis (Fig. 5j).

Effect of LINC00518 in LUAD cells in vivo

First, a xenograft mouse model was used to confirm the effect of LINC00518 in LUAD cells *in vivo*. As shown in illustration (Fig. 6a, b), tumours formed by LINC00518 knockdown cells were significantly smaller in size than those formed by the contrast cells. Tumour weights, in accordance with these results, were found to be lighter in cells with LINC00518 knockdown (Fig. 6c). Next, we resected the tissues from the xenograft tumours, and analyzed them to verify LINC00518, and CTHRC1 by Western blotting and qRT–PCR (Fig. 6d, e).

Down-regulation of miR-335-3p reverses the inhibition of cell proliferation, migration, invasion, and colony formation induced by LINC00518-knockdown

Transfection of A549 and H1299 cells with sh-LINC00518 reduced cell proliferation while inhibiting miR-335-3p reversed this effect (Fig. 7a). Additionally, it was identified that the cell survival capability was remarkably decreased in the LINC00518-silenced cells, by colony formation assays, while this negative impression on cell survival could be reversed by the low expression of miR-335-3p (Fig. 7b **and Additional file 5: Fig. S1b)**. Besides, LINC00518 got in touch with regulating invasion and migration in LUAD cells, suggested by Transwell assays, and miR-335-3p inhibitors reversed LINC00518 knockdown-induced suppression of migration and invasion (Fig. 7c **and Additional file 5: Fig. S1c)**. The flow cytometry results also showed that the impact on the cell cycle of LINC00518 knockdown in A549 and H1299 cells could be reversed by miR-335-3p inhibitors (Fig. 7d). Knockdown the expression of LINC00518 in A549 and H1299 cells decreased protein levels of CTHRC1 were confirmed by Western blotting, while miR-335-3p inhibitors reversed this effect (Fig. 7e). A schematic of this search is shown in Fig. 8.

Discussion

Located on human chromosome 6p24.3, the expression of long intergenic nonprotein coding RNA 518 (LINC00518) is significantly up-regulated in some tumour tissues [9, 11, 13]. LINC00518, indicated from the published studies, might be a crucial cancer gene and take an indispensable part in the occurrence and development of tumours through various biological mechanisms. Nevertheless, the function of LINC00518 in the progression of lung adenocarcinoma has not yet been clarified.

As crucial regulators in multiple cellular processes associated with tumorigenesis and metastasis, in the past decades, lncRNAs have drawn great much attention and interest [39, 40]. In the present study, we noted that LINC00518 expression was meaningfully up-regulated in LUAD tissues contrasted with normal lung tissues and that patients had a markedly poor prognosis in terms of OS, PFS, DFS, and DSS with high expression of LINC00518. This shows that LINC00518 might be a potential biomarker for the diagnosis and prognosis and a molecular target of patients with LUAD. Moreover, the proliferation and clonogenicity of LUAD cells could be increased by LINC00518, migration and invasion could be induced, and the cell cycle could be affected. It might suggest that LINC00518 serves as an oncogene in LUAD cells.

Serving as miRNA sponges, LINC00518 regulates the target genes of miRNAs, basically localized in the cytoplasm, thereby inhibiting miRNAs' functions [13–15]. Hence, we investigated the miRNAs that bind to

LINC00518. It was verified in this study that LINC00518 could directly target miR-335-3p, which, in turn, target CTHRC1. In other words, LINC00518 upregulates the expression of CTHRC1 by binding competitively to miR-335-3p.

Next, we demonstrated that LINC00518 could specifically inhibit the expression of miR-335-3p or not. Our previous study showed that up-regulating miR-335-5p expression could inhibit the proliferation and migration of NSCLC cells [37]. Accordingly, we considered that miR-335-3p, homologous with miR-335-5p, played the same role in LUAD. To verify the specific function of miR-335-3p in LUAD cells, we performed malignant phenotype assays. We detected that miR-335-3p could take a part in an anti-oncogene by inhibiting the activity of CTHRC1 in LUAD. Our investigation results showed that miR-335-3p might serve as a tumour suppressor, which has tremendous significance in the development of new targeted therapies for LUAD.

Although the function of CTHRC1 in LUAD is well-known, the mechanisms remained unclear. Research conducted before showed that expression of CTHRC1 is associated with prognosis and can act as an important predictor of progression-free survival (PFS) and overall survival (OS) in LUAD [38]. CTHRC1 could induce the invasion ability of NSCLC by upregulating MMP-7/MMP-9 [41]. In our study, we verified that in LUAD cells the ability of cell proliferation, migration and invasion could be increased by CTHRC1.

Integrins could be classified into four, including collagen receptors, laminin receptors, leukocyte-specific integrins, and receptors recognizing Arg-Gly-Asp (RGD) peptide motifs[42, 43]. There are reports of CTHRC1, which could accelerate migration and adhesion by upregulating the expression of the integrin β family, and the phosphorylation of focal adhesion kinase (FAK) in ovarian cancer, hepatic carcinoma, and pancreatic cancer [28, 44, 45]; however, it has not been found in lung cancer yet. As a crucial cell movement regulator, FAK could be stimulated via phosphorylation by transmembrane integrins and varieties of growth factors, connecting to the formation and turnover of focal adhesions, which then phosphorylated and induce downstream pathway signalling[46–48]. It has been reported that ligation of αVβ3 and αvβ5 integrins could mediate FAK activity [49]. In our previous research, integrin αVβ3 could promote cell proliferation in NSCLC by activating the downstream FAK/AKT and ERK signalling pathways [50]. We used online database functional analysis to find that LINC00518 and CTHRC1 could be clustered on FAK signalling and CTHRC1 co-expressed with integrin β3 in LUAD.

Conformably, our research indicated that the low-expression of CTHRC1 influences the expression of integrin β3, and the phosphorylation of FAK. Moreover, we gave a piece of evidence that CTHRC1 physically interacts with integrin β3, which further attests to the mechanism of CTHRC1 in LUAD. In the study, there are reasonable associations between integrins and CTHRC1 in LUAD, the results showed that CTHRC1 could regulate FAK signalling by interacting with integrin αVβ3 complexes. Whereas, it remains to further detect the complicated mechanism of the CTHRC1-integrin complex.

Previous studies have demonstrated that targeting the integrin β3/FAK signalling could enhance the anti-tumour activity and attenuate cancer metastasis in melanoma, endometrial cancer, NSCLC, and ESCC [51–55]. Consequently, the FAK-inhibitor VS-6063 was used to verify whether the inhibition of FAK

phosphorylation would work in conjunction with the knockdown of LINC00518 on LUAD cell proliferation. Moreover, overexpression of CTHRC1 could reverse the LUAD cell growth inhibition induced by VS6063 treatment. Hence, we proved that CTHRC1 can affect integrin β3/FAK signalling and broaden the application of the FAK-inhibitor VS-6063 in LUAD which might shed light on clinical treatment. Interestingly, the FAK-inhibitor reduced the expression of CTHRC1 when miR-335-3p was inhibited and CTHRC1 was over-expressed. We suspect that there is positive feedback between CTHRC1 and FAK signalling. In summary, our results provide the first evidence that CTHRC1 interacts with integrin β3 and accelerates FAK phosphorylation in LUAD.

To sum up, we clarified the clinical significance and biological functions of a cytoplasmic lncRNA, LINC00518, in LUAD. LINC00518, as a molecular sponge to regulate CTHRC1, promoted LUAD growth and migration through integrin-mediated focal adhesion signalling. Furthermore, the expression of LINC00518 is high in LUAD samples than in normal, and the expression of LINC00518 is an indicator of prognosis in LUAD patients. The LINC00518 /miR-335-3p /CTHRC1 axis might broaden the application of the FAK-inhibitor VS-6063 in LUAD. Given the above, our results demonstrated that LINC00518 could work as a promising biomarker of prognosis and synergetic therapeutic target of FAK-inhibitor in LUAD.

Declarations

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Author contributions

Z.L., Y.Z., J.Z., and J.H. designed and supervised the study. R.S, X.C., and D.S. performed most of the experiments and wrote the manuscript. R.Z. and Y.Z. helped to conduct experiments in vivo. A.W. and W.Z. helped to edit the manuscript. Y.L. was responsible for statistical analysis. All authors read and approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article and its additional files.

Consent for publication

Not applicable.

Competing interest

The authors have no conflicts of interest.

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Figures

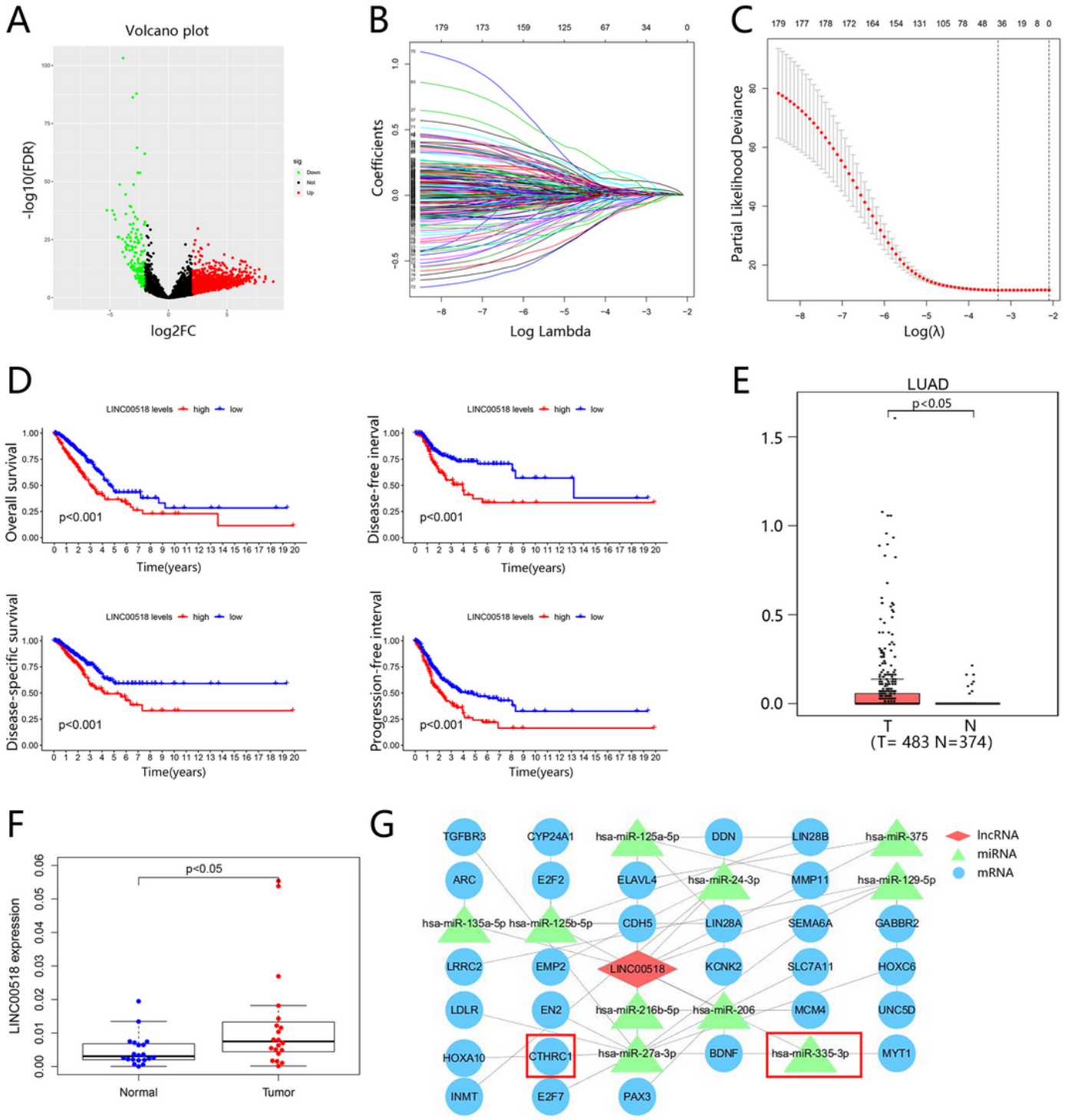

Figure 1

LINC00518 was up-expressed in LUAD and a venturesome factor for the survival of LUAD. (A) The volcano map shows all lncRNAs with differential expression in LUAD from TCGA. (B) Lambda of the LASSO regression of differentially expressed lncRNAs. (C) The minimum lambda included 39 lncRNAs. (D) The OS, PFS, DFS, and DSS of LUAD patients with high or low expression of LINC00518. Computed p-value by using the log-rank test. (E) TCGA data shows that LINC00518 is upregulated in LUAD compared with normal tissues. (F) The expression of LINC00518 was shown by qPCR in 20 paired LUAD tissues and their corresponding adjacent noncancerous lung tissues in our study. (G) Cytoscape was used to provide the visual interaction of LINC00518–miRNA–target gene.

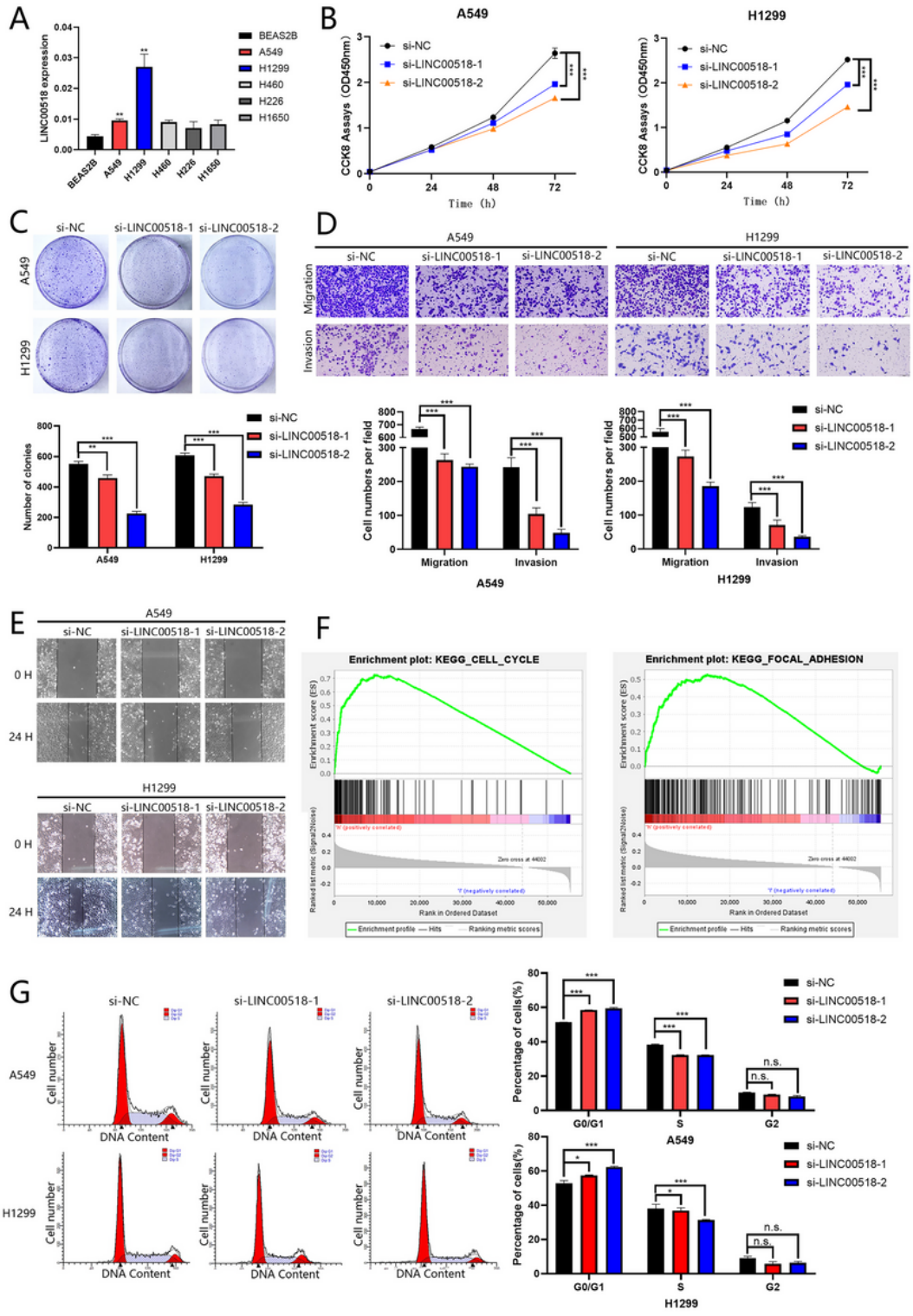

Figure 2

Knockdown of LINC00518 suppressed cell proliferation and metastasis, and reduced viability. (A) Expression level of LINC00518 in human bronchial epithelial cells (BEAS-2B) and lung cancer cell lines(A549, H1299, H460, H226, H1650). (B, C) CCK-8 assay and Colony formation analysis detected the ability of cell proliferation of A549/H1299 cells transfected with si-LINC00518 or NC. (D, E) Transwell assay and Wound-healing assay showing the effect on LUAD cell metastasis capability following

LINC00518 knockdown compared with the control. (F) Kegg pathway analysis identified that LINC00518 might take a significant part in the cell cycle and focal adhesion. (G) Cell cycle distributions were analyzed in A549/H1299 cells. All results indicate SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

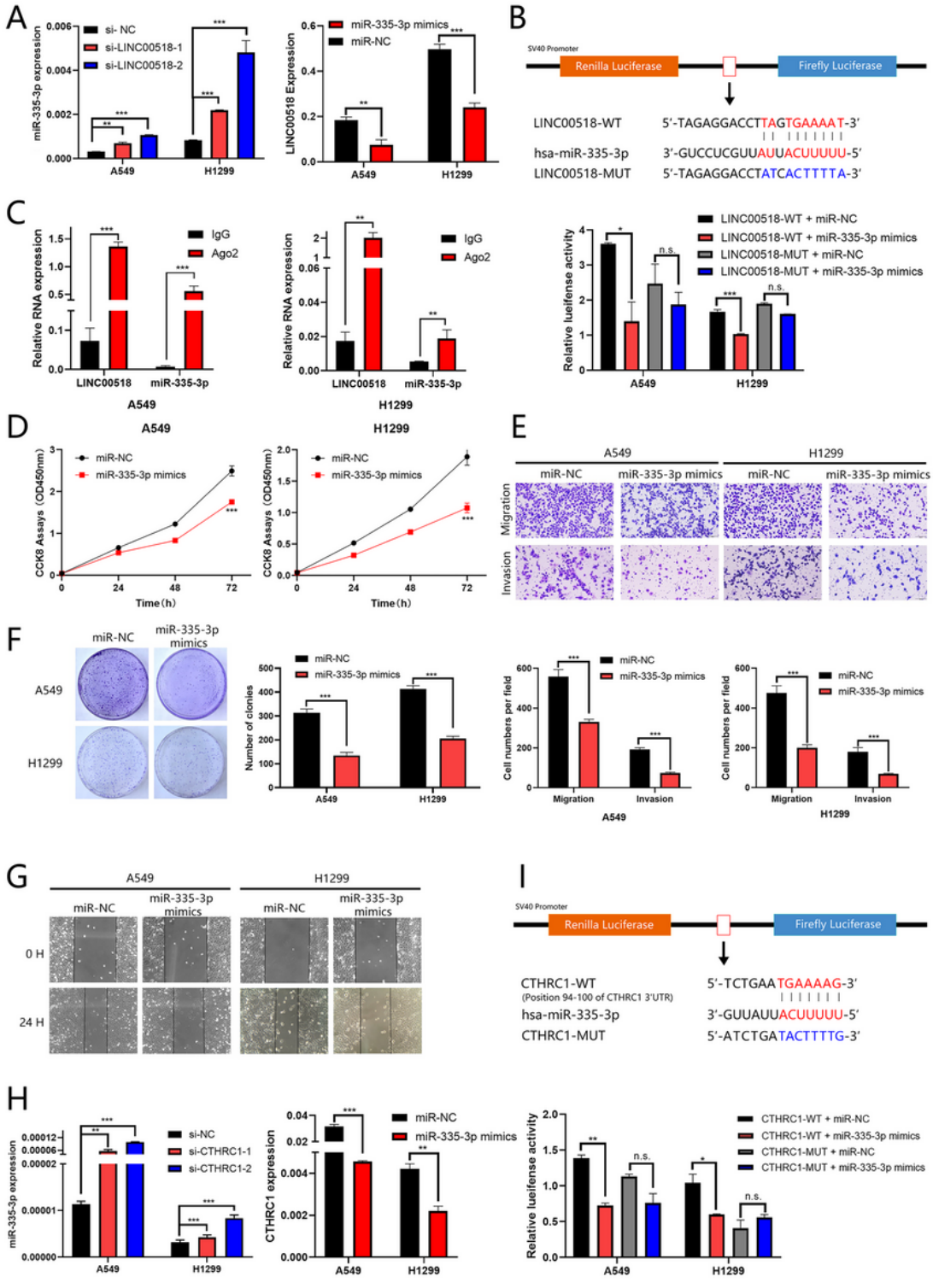

Figure 3

LINC00518 directly targets miR-335-3p, promoting CTHRC1 expression and miR-335-3p suppresses LUAD cell proliferation and metastasis (A, H) Detection of the expression of miR-335-3p in A549/H1299 cells

following transfection with LINC00518 siRNA, CTHRC1 siRNA, or NC and, in turn, the expression of LINC00518 or CTHRC1 following transfection with miR-335-3p mimics or NC. (B, I) The dual-luciferase assay showed that the relative dual-luciferase activity of the LINC00518-WT or CTHRC1-WT group was directly inhibited by miR-335-3p mimics compared with that of the control group. (C) RIP assay was used to further make sure the direct interaction between LINC00518 and miR-335-3p. (D, F) As is shown in the CCK-8 assay and colony formation analysis that transfected with miR-335-3p mimics inhibited the capability of cell proliferation of A549/H1299 cells. (E) Transwell assay and Wound-healing assay showing that miR-335-3p mimics in A549/H1299 cells can significantly inhibit the ability of cell metastasis compared with the cell following transfection with NC. All results indicate SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

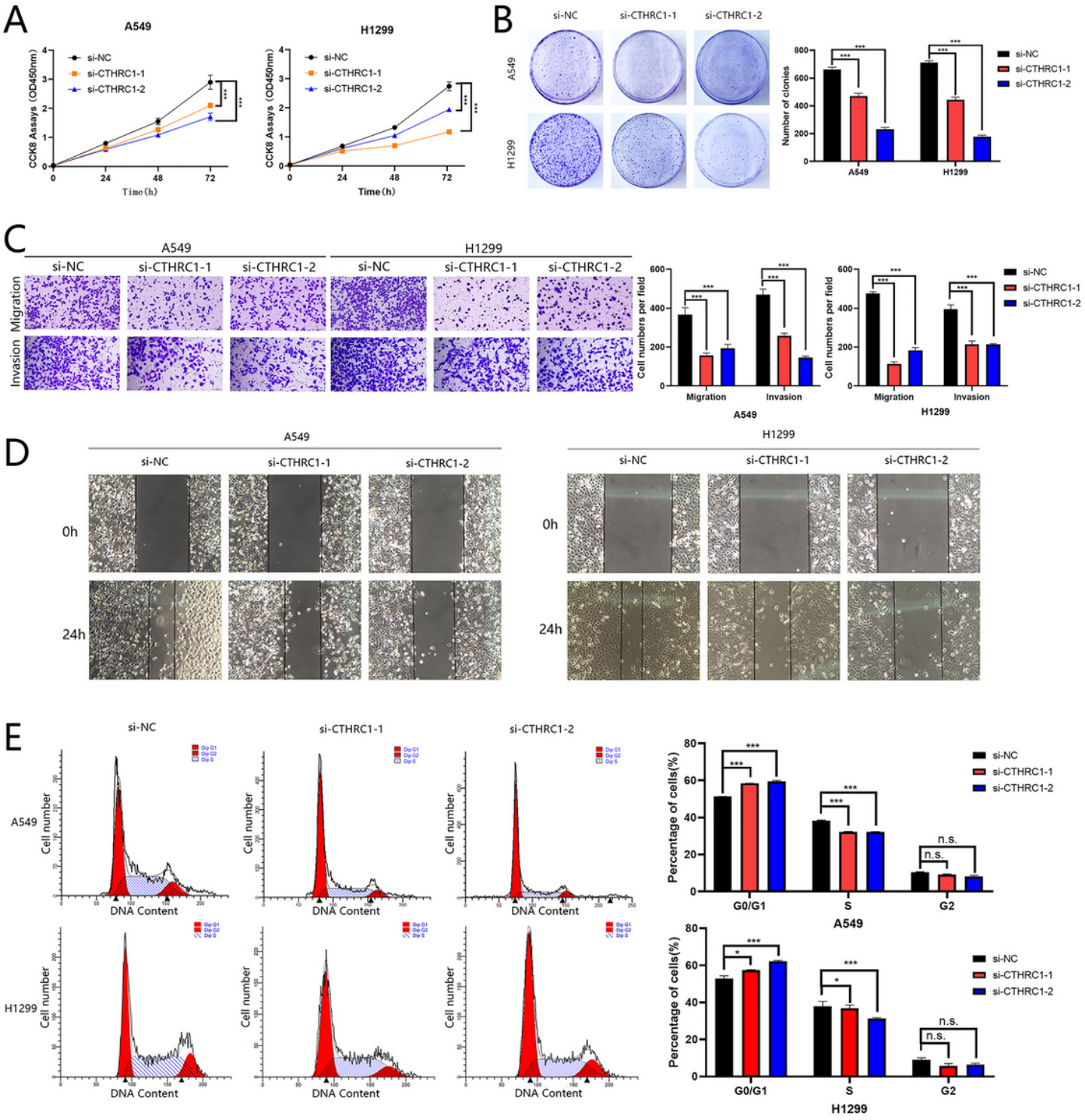

Figure 4

Knockdown of CTHRC1 suppressed cell proliferation and metastasis, and reduced viability. (A, B) The capability of cell proliferation of A549/H1299 cells transfected with si-CTHRC1 or NC was detected by CCK-8 assay and colony formation analysis. (C, D) Knockdown of CTHRC1 in A549/H1299 cells can meaningfully inhibit cell metastasis ability compared with the control were showed by Transwell assay

and Wound-healing assay. (E) Cell cycle distributions were analyzed in A549/H1299 cells. All results indicate SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

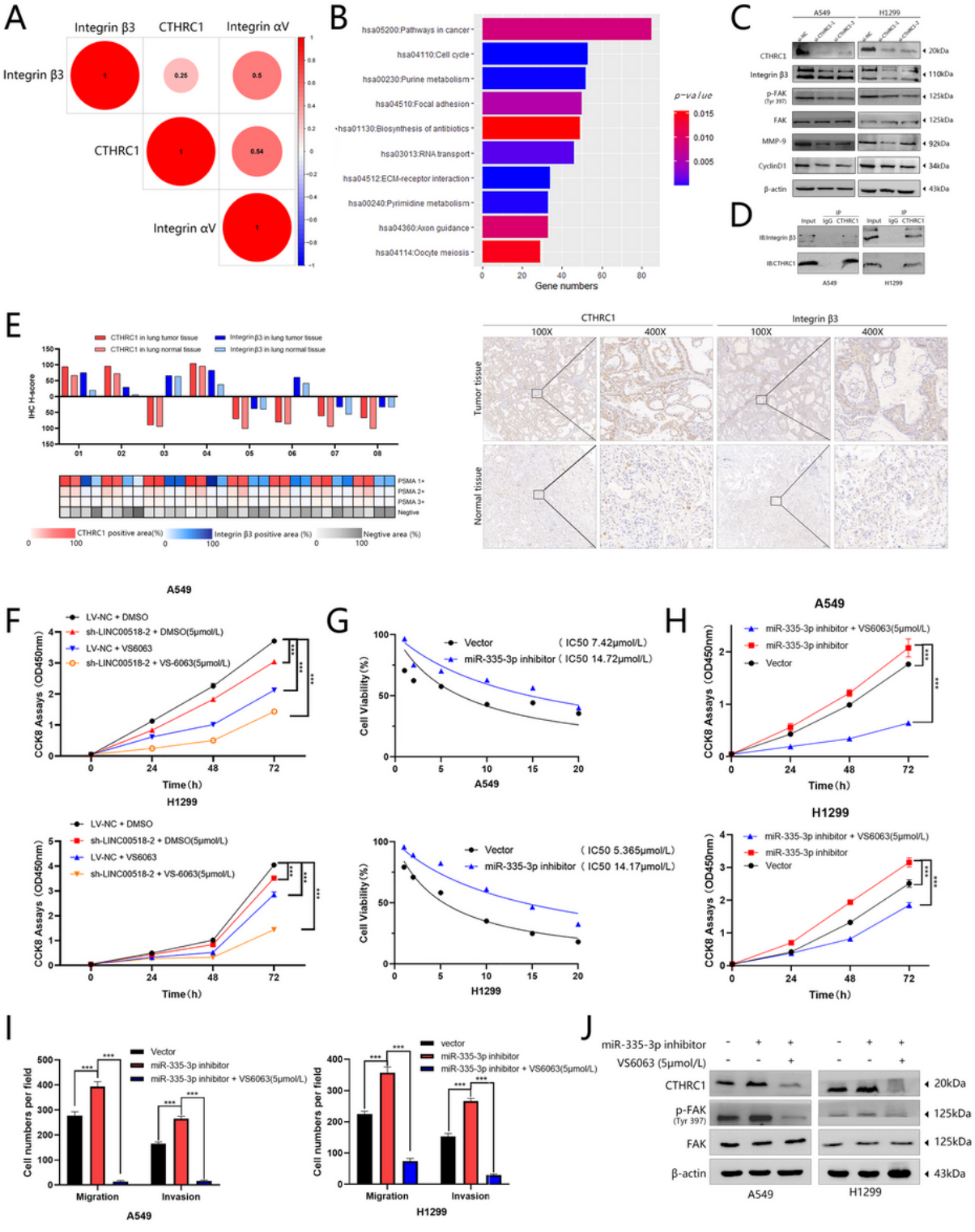

Figure 5

CTHRC1 could affect integrinβ3/FAK signalling (A) The correlation of CTHRC1 and integrin αVβ3 from the TCGA database. (B) Pathway analysis was applied to investigate the remarkable pathways of the differentially expressed genes according to the TCGA database. Then we chose the DAVID database for

functional analysis. (C) Western blotting analysis showed that integrin β3, p-FAK, FAK, MMP-9, and CyclinD1 protein levels were regulated by CTHRC1. (D) Co-immunoprecipitation of CTHRC1 and integrin β3 in LUAD cells exposed that endogenous integrin β3 in LUAD cells was immunoprecipitated by CTHRC1 antibody. (E) The co-expression between CTHRCI and integrin β3 was shown by IHC of sections from the patients (scale bar, 100 μm, 20 μm). (F) CCK-8 assays were applied to detect the cell with VS6063 (5μmol/L) had notably lower capability of cell proliferation than the control cells with DMSO (5μmol/L). (H) The IC50 of A549 and H1299 cells, which inhibited miR-335-3p. (I) CCK-8 assay showed the proliferation of A549/H1299 cells that inhibited miR-335-3p transfection with VS6063 (5 μmol/L). (I) Transwell assays showed that miR-335-3p inhibited cells migration and invasion following transfection as described previously. (J) Western blotting analysis showed that CTHRC1 and p-FAK protein levels were induced by a FAK-inhibitor when miR-335-3p was inhibited. All results indicates SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

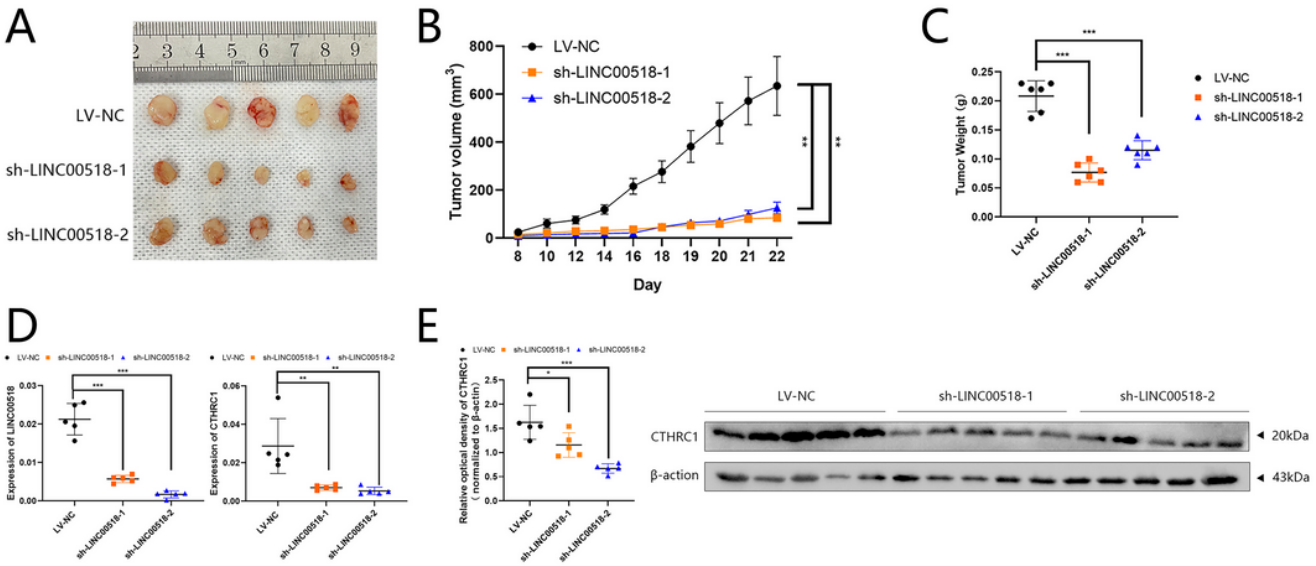

Figure 6

Knockdown of LINC00518 inhibits LUAD tumour growth in vivo. (A-C) As shown in the transplantation tumour experiments that repression of LINC00518 expression using the shLINC00518 plasmid reduced the tumorigenic ability and tumour volume of A549 cells could be observed. (D) The expression level of LINC00518 and CTHRC1 in tumour tissues of mice was detected by qRT–PCR. (E) As is shown in Western blotting that LINC00518 expression or treatment of the cells with LV-NC decreased CTHRC1 protein expression in tumour tissues of mice. All results indicate SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

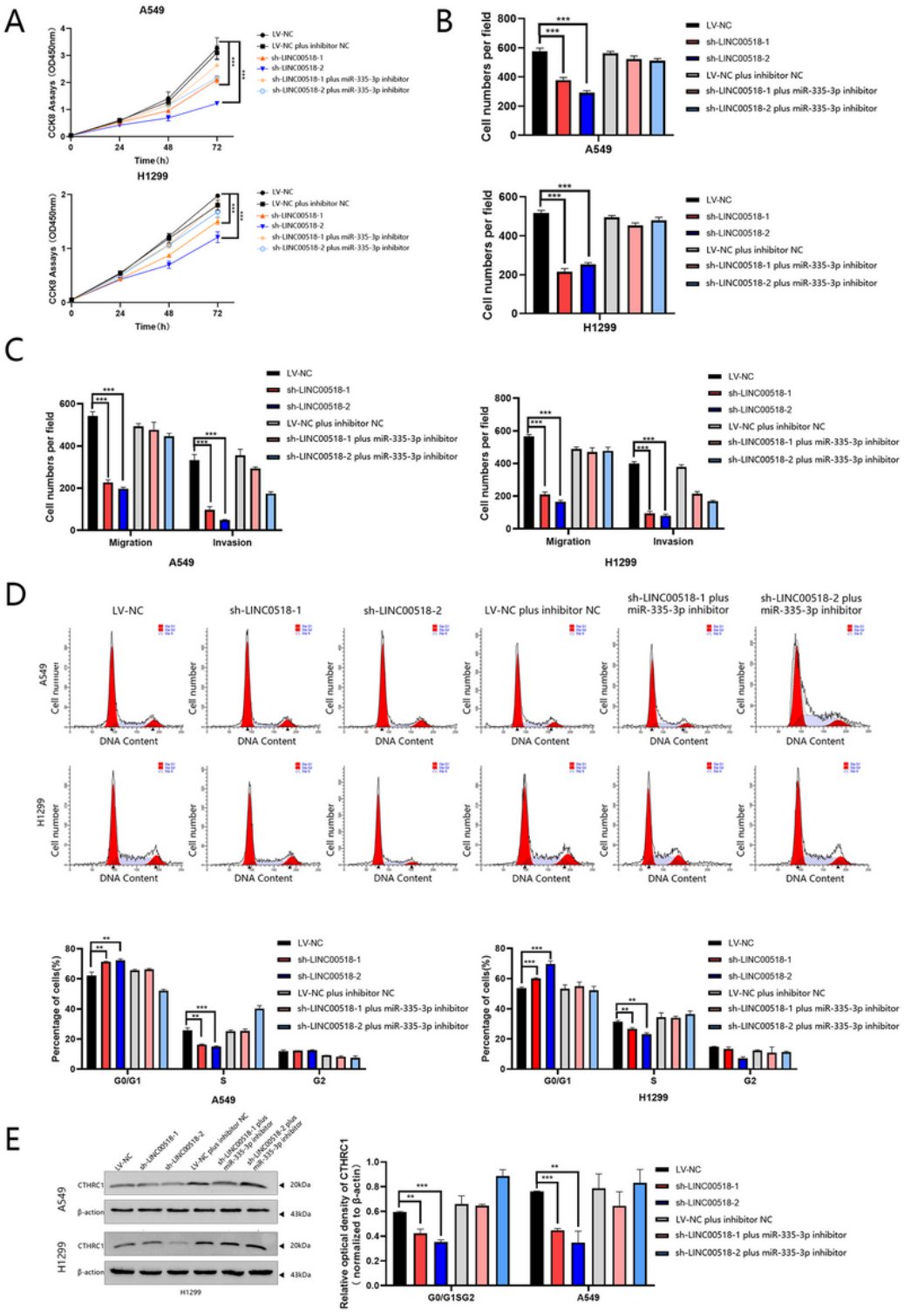

Figure 7

LINC00518 promotes LUAD cell proliferation and metastasis through the miR-335-5p/CTHRC1 axis. (A-B) CCK-8 assay and colony formation analysis were applied to detect the proliferation of A549/H1299 cells transfected with LINC00518 shRNA, LINC00518 shRNA plus miR-335-3p inhibitor, or the control. (C) As is shown in the Transwell assay the effect on LUAD cell migration and invasion following transfection is described before. (D) Cell cycle distributions were analyzed in A549 and H1299 cells by flow cytometry.

(E) Western blots identified the protein expression changes in LINC00518 shRNA and LINC00518 shRNA plus miR-335-3p inhibitor transfected LUAD cells. β-actin was used as a control. All results indicate SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

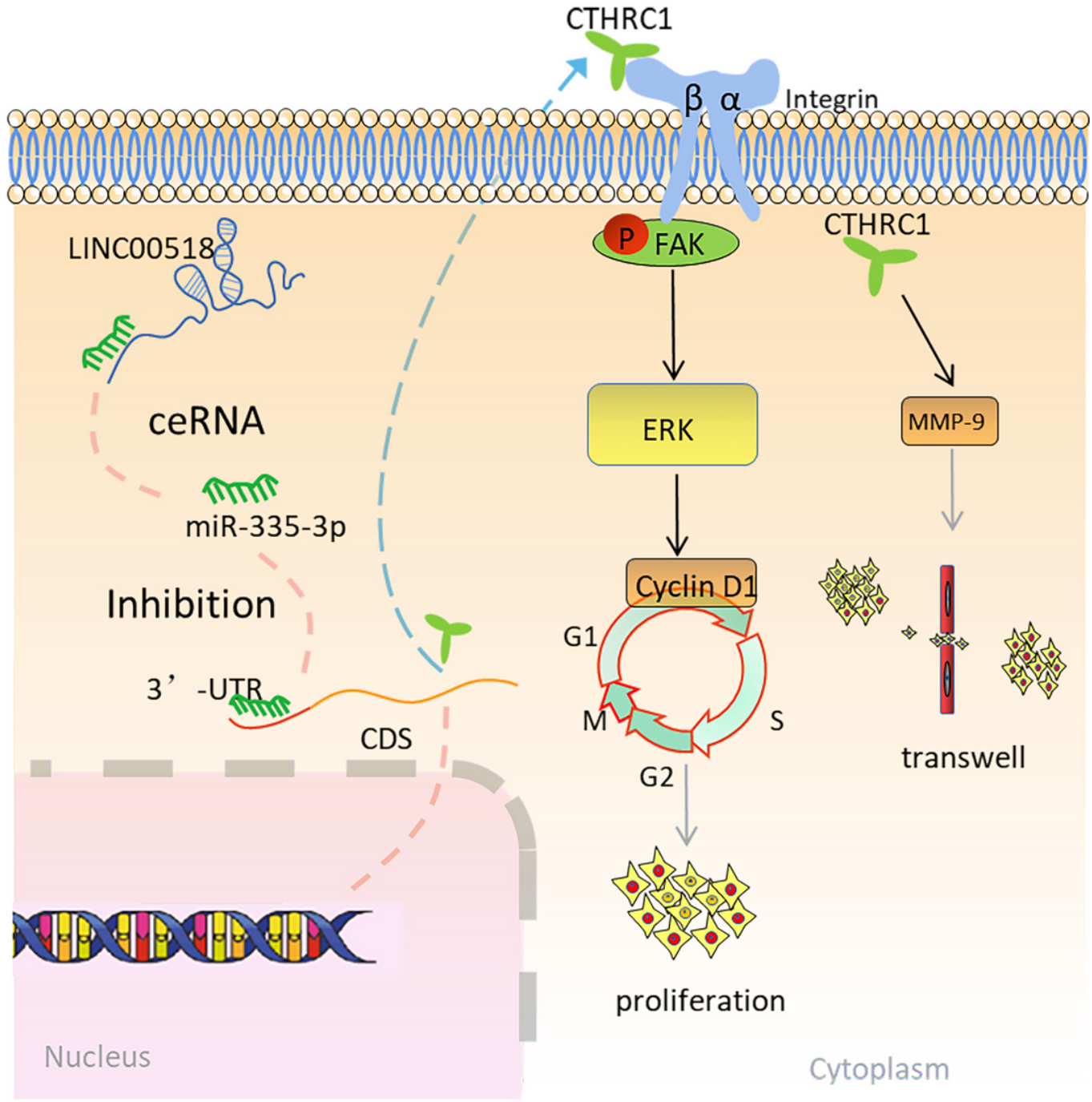

Figure 8

The model of the LINC00518/miR-335-3p/CTHRC1 axis is in the control of the integrin β3/FAK signalling pathway.

Supplementary Files

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