Cortex Mori extract inhibits migration and invasion of lung adenocarcinoma cells by blocking *RECQL4*-induced NF-κB and ERK signaling pathways

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ORIGINAL ARTICLE

Abstract

Lung adenocarcinoma (LUAC) is one of the usual tumors of the lung with high mortality rate. RecQ-like helicase 4 (*RECQL4*) gene has been discovered to take part in the progression of different cancers by undertaking as an oncogene, and is relevant with poor prognosis of LUAC. Cortex Mori (CM) extract has been investigated to affect cellular progress to regulate different diseases. However, the detailed functioning of *RECQL4* and CM extract, as well as their regulatory mechanisms in LUAC, has not been illustrated. The purpose of the present study was to probe the impact of *RECQL4* and CM extract on progression of LUAC. The expression of *RECQL4* in LUAC was assessed by The Cancer Genome Atlas (TCGA) database. The mRNA expression of *RECQL4* was examined by real-time quantitative polymerase chain reaction. The protein expressions (epithelial–mesenchymal transition [EMT] process, nuclear factor *kappa B* [NF-κB] and extracellular signal-regulated kinase [ERK] signaling pathways-related proteins) were determined by Western blot analysis. The cell proliferation was tested through cell counting kit-8 assay. Cell migration and invasion was affirmed by wound-healing and transwell assays. The cell senescence was assessed through senescence-associated beta-galactosidase staining. The cell cycle was inspected by flow cytometry. Our findings demonstrated that *RECQL4* exhibited higher expression in LUAC tissues and cell lines. Through functional experiments, we found that *RECQL4* facilitated cell proliferation, migration, and invasion as well as EMT progression. In addition, *RECQL4* relieved cell cycle arrest and cell senescence. Moreover, *RECQL4* activated NF-κB and ERK signaling pathways by enhancing phospho(p)-p65–p65 and p-ERK–ERK levels in LUAC. CM extract exhibited antitumor effects in LUAC, and blocked *RECQL4*-induced NF-κB and ERK signaling pathways. Our results manifested that CM extract inhibited migration and invasion of LUAC cells by blocking *RECQL4*-induced NF-κB and ERK signaling pathways. This result could provide a promising therapeutic strategy for LUAC.

Keywords: Cortex Mori (CM) extract; *RECQL4*; lung adenocarcinoma; NF-κB and ERK signaling pathways

Introduction

Lung cancer, the most hackneyed type of cancer, is one of the leading causes of deaths globally (Nasim *et al*., 2019; Romaszko and Doboszyńska, 2018). In China, the occurrence and mortality rate because of lung cancer are rising rapidly and is on peak in both males and females (Jones and Baldwin, 2018; Wu *et al*., 2021). Non-small cell lung cancer (NSCLC) is the major type of lung cancer (about 85%) which includes lung adenocarcinoma (LUAC) and lung squamous cell carcinoma (LUSC) (Herbst *et al*., 2018). At present, the most effective treatment for early LUAC patients are mediastinal lymph node dissection and anatomical surgical resection (Denisenko *et al*., 2018). The advanced LUAC patients have diversified disease manifestations, and thus need complex multimodal

treatments, including systemic treatment and local treatment (chemotherapy, radiotherapy, etc.; Succony *et al*., 2021). In recent years, although targeted therapies have been developed to prolong the survival period of cancer patients, the 5-year survival rate for lung cancer patients has been consistently low over the past 20 years (Hirsch *et al*., 2017; Lemjabbar-Alaoui *et al*., 2015). Therefore, finding new biological targets is essential for the early diagnosis and cure of lung cancer as well as enhancing the survival period of the patients.

RecQ-like helicase 4 (*RECQL4*) gene is a member of RECQ helicase family and combines in maintaining the genomes' stability of nucleus and mitochondria (Balajee, 2021; Croteau *et al*., 2012). *RECQL4* gene mutations are associated with three human autosomal recessive disorders, including Rothmund–Thomson syndrome, RAPADILLINO syndrome, and Baller–Gerold syndrome (Kitao *et al*., 1999; Siitonen *et al*., 2003; Van Maldergem *et al*., 2006). Recent reports have indicated that *RECQL4* is highly expressed in gastric cancer and enhances cisplatin resistance through modulating the Ak strain transforming–Y box binding protein 1–multidrug resistance protein 1 (AKT–YB1–MDR1) signaling pathway (Mo *et al*., 2016). In addition, *RECQL4* also exhibits higher expression in esophageal squamous cell carcinoma, modulates nuclear factor *kappa B* (NF-κB) and extracellular signal-regulated kinase (ERK) signaling pathways, and plays a pivotal role in regulating DNA damage responses, redox homeostasis, and cell survival (Lyu *et al*., 2021). *RECQL4* stimulates ovarian cancer cell proliferation and invasion through regulating *MAFB* (MAF bZIP transcription factor B) gene expression (Guo *et al*., 2020). It has been illustrated that *RECQL4* is in relation to poor prognosis of LUAC (Jiang *et al*., 2021). In the present study, through TCGA database, we discovered that the *RECQL4* expression in LUAC tissues exists higher than that in normal tissues. Nonetheless, the roles of *RECQL4* in LUAC remain unclear.

Therefore, this study mainly explored the role and specific regulatory mechanism of *RECQL4* in LUAC. In conclusion, our findings unveiled that Cortex Mori (CM) extract inhibited migration and invasion of LUAC cells by blocking *RECQL4*-induced NF-κB and ERK signaling pathways. This study highlighted that *RECQL4* may be a helpful bio-target for CM extract treatment in LUAC.

Materials and Methods

Bioinformatic analysis

The *RECQL4* expression in normal or primary tumor tissues was analyzed from TCGA database through the UALCAN online platform (http://ualcan.path.uab.edu/). The survival rate of LUAC patients with low or high *RECQL4* expression was evaluated through the Kaplan–Meier plotter database (https://kmplot.com/analysis/).

Cell lines and cell culture

The NSCLC cell lines (A549, PC-9, HCC827, and NCI-H2009) and human normal lung epithelial cell line (BEAS-2B) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). RPMI-1640 medium (Procell Life Science & Technology, China) was supplemented with 10% fetal bovine serum (FBS; Procell), 1% GlutaMax (Procell), and 1% penicillin–streptomycin solution (100×; Procell). The cells were cultured in supplemented RPMI-1640 medium at 37°C with 5% CO_2.

In the present study, CM (5 mg/mL; Tianjiang Pharmaceutical, China) was applied to treat A549 and PC-9 cells for 24 h.

Transfection

Small interfering RNA (siRNA) for knocking down of *RECQL4* (si*RECQL4*) and its negative control (siNC) were acquired from GenePharma (Shanghai, China). The plasmid cloning (pc)DNA3.1 was designed to overexpress *RECQL4* (*RECQL4*), and its negative control (pcDNA3.1) was also acquired in the same manner. The transfection of these plasmids in LUAC cells was done through Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA).

RECQL4 siRNA: 5′-CGGAAGAAAGGGGAGTGTTT TGG-3′. The sequence of the plasmid overexpression of *RECQL4* is shown in Supplementary 1.

Real-time quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (Invitrogen) was used to gain total RNA from LUAC cells. The reverse transcription of RNA to complementary DNA (cDNA) was performed through SuperScript™ II reverse transcriptase kit (Invitrogen). Next, RT-qPCR was carried out through the SYBR Premix Ex Taq™ (Takara, Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was deemed as an internal reference, and the data quantification was performed with $2^{-\Delta\Delta Ct}$ method.

The primer sequences (5′-3′) were displayed as follows: *RECQL4*-forward: GAT CCT GGC TGG TTA CAG CG; *RECQL4*-reverse: AGT TGT GAT TCC TCT GAG CCT; GAPDH-forward: GTC AGC CGC ATC TTC TTT TG; and GAPDH-reverse: GCG CCC AAT ACG ACC AAA TC.

Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 10% was executed for the segregation of extracted proteins (40 μg), which were then placed to polyvinylidene difluoride (PVDF) membranes (Millipore). Next, after sealing, the membranes were mixed with primary antibodies overnight at 4°C against *RECQL4* (1:1,000, ab188125, rabbit; Abcam, Cambridge, UK); E-cadherin (1:1,000, ab1416, rabbit); N-cadherin (1:1,000, ab76011, rabbit); snail (1:1,000, ab180714, rabbit); slug (1:1,000, ab180714, rabbit); vimentin (1:1,000, ab92547, rabbit); phospho(p)-p65 (1:1,000, ab76302, rabbit); p65 (1:1,000, ab16502, rabbit); phospho (p)-ERK (1:1,000, ab201015, rabbit); ERK (1:1,000, ab184699, rabbit); and GAPDH (1:1000, ab8245, mouse, the internal reference). Afterward, appropriate secondary antibodies (1:2,000, ab7090, goat anti-rabbit immunoglobulin G [IgG] H&L (HRP) pre-adsorbed; Abcam) were mixed into membranes at room temperature for 1 h incubation. Finally, the assessment of bands was done through chemiluminescence detection kit (Thermo Fisher Scientific, MA).

Cell Counting Kit-8 (CCK-8) assay

At first, the LUAC cells cultured in 96-well plate at 24, 48, and 72 h were mixed with CCK-8 reagent (20 μL; Sigma-Aldrich, St. Louis, MO). The assessment of the OD value was done at 450 nm, post-incubation of 4 h, under microplate reader (SpectraMax M5, Molecular Devices, San Jose, CA).

Wound healing assay

A549 and PC-9 cells were plated into a six-well plate. Next, a wound was created by tip of a 200-μL pipette. After gently rinsing with phosphate-buffered saline solution (PBS), the cells were mixed with serum-free medium. Images of the wound were obtained at 0 and 24 h under a light microscope (Olympus Corporation, Tokyo, Japan).

Transwell assay

The Matrigel, a gelatinous protein mixture (Becton Dickinson, USA)-coated Transwell chambers (Corning Life Sciences, Corning, NY) were applied for invasion assay. Briefly, cells (1×10^4) and Dulbecco's modified eagle *medium* (DMEM) without FBS were placed in the upper chamber, and DMEM with 10% FBS was placed in the lower chamber. Then, the fixing (90% ethanol) and dyeing (0.1% crystal violet) of the invaded cells were performed. The invaded cells were counted through inverted light microscope (Olympus Corporation):

The cell invasion rate = (Invaded cells/Total cells) ×100%.

Flow cytometry (for cell cycle)

Cells were first fixed in 70% cold alcohol. After washing, staining of cells was performed with propidium iodide (PI; Beijing Solarbio Science & Technology, Beijing, China). The cell cycle phases (G1 [Gap 1 phase], S [synthesis phase], and G2 [Gap 2 phase]) were assessed under a FACS caliber flow cytometer (BD Bioscience, San Diego, CA).

Senescence-associated beta-galactosidase (SA-β-gal) staining

Fixing with 4% paraformaldehyde (Beyotime Institute of Biotechnology, Shanghai, China) was done for A549 and PC-9 cells. SA-β-gal activity was examined through β-galactosidase staining kit (Beyotime Institute of Biotechnology). At pH 6.0, 4-methylumbelliferyl β-D-galactopyranoside (MUG) was converted into 4-methylumbellifer-one (4-MU), a fluorescent hydrolysis product, and the SA-β-gal activity was verified to assess cell senescence. Images were observed using a light microscope (Olympus Corporation).

Statistical analysis

The SPSS 20.0 software was used for statistical analysis. All data were expressed as mean ± standard deviation (SD). Student's *t*-test (between two groups) or one-way ANOVA (for multiple groups) was applied for statistical comparisons. All experiments were performed in triplicate; $P < 0.05$ was considered statistically significant.

Results

RECQL4 possessed higher expression in LUAC

At first, it was discovered that *RECQL4* exhibited higher expression in LUAC tissues in TCGA database through UALCAN online platform (http://ualcan.path.uab.edu/) (Figure 1a). Through the Kaplan–Meier plotter database (https://kmplot.com/analysis/), LUAC patients with high *RECQL4* expression have worse survival rate than that of low *RECQL4* expression (Figure 1b). Additionally, the enhanced mRNA expression of *RECQL4* was uncovered

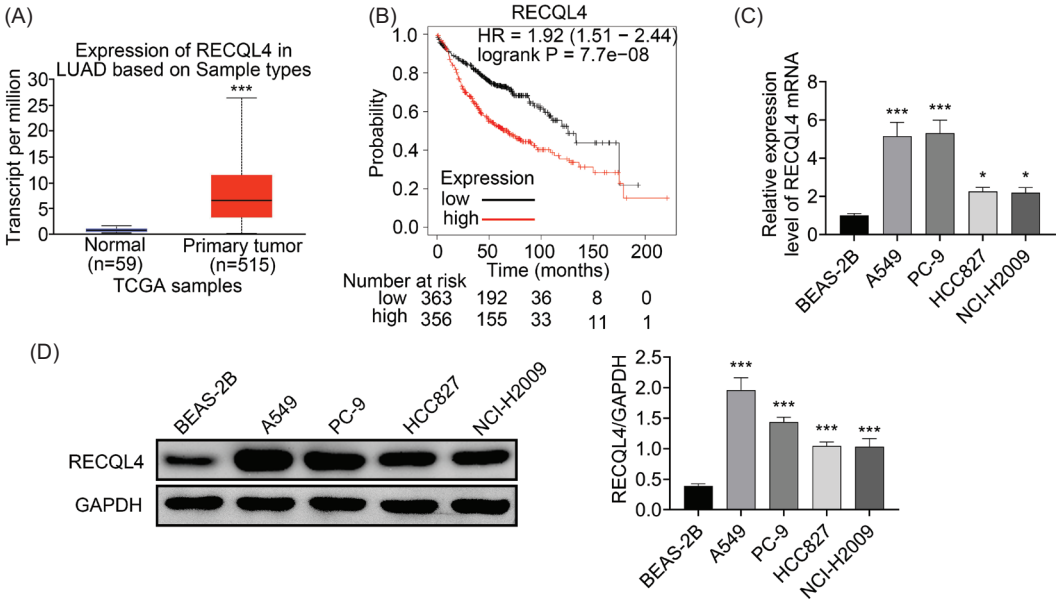

Figure 1 ***RECQL4*** **exhibited higher expression in LUAC. (A) The expression of** ***RECQL4*** **in LUAC was assessed by TCGA database. (B) The survival rate of LUAC patients with low or high** ***RECQL4*** **expression was evaluated through Kaplan–Meier plotter database. (C) The mRNA expression of** ***RECQL4*** **was tested through RT-qPCR in LUAC cell lines (A549, PC-9, HCC827, and NCI-H2009) and the normal lung epithelial cells (BEAS-2B). (D) The protein expression of** ***RECQL4*** **was tested by Western blot analysis. ****P* **< 0.05, ******P* **< 0.001.**

in LUAC cell lines (A549, PC-9, HCC827, and NCI-H2009; Figure 1c). Similarly, the *RECQL4* protein expression was also increased in LUAC cell lines (Figure 1D). To sum up, *RECQL4* exhibited higher expression in LUAC.

RECQL4 enhanced cell proliferation

The knockdown and overexpression efficiencies of *RECQL4* mRNA and protein levels were confirmed as displayed in Figures 2a and 2b. Furthermore, it was demonstrated that the cell viability decreased after silencing *RECQL4* and increased after overexpressing *RECQL4* (Figure 2c). This finding suggested that *RECQL4* enhanced cell proliferation.

RECQL4 facilitated cell migration and invasion as well as epithelial–mesenchymal transition (EMT) progression

Findings revealed that both cell migration and invasion were suppressed by *RECQL4* inhibition but strengthened by *RECQL4* overexpression (Figures 3a and 3b). In addition, the E-cadherin protein expression was enhanced after repressing *RECQL4*, but it was reduced after overexpressing *RECQL4*. Meanwhile, the N-cadherin, snail, slug, and vimentin were decreased after *RECQL4* suppression but increased after *RECQL4* overexpression (Figure 3c). These data indicated that *RECQL4* facilitated EMT progression.

RECQL4 relieved cell cycle arrest and cell senescence

It was demonstrated by flow cytometry that the cell cycle was retarded at G1 phase after *RECQL4* knockdown but promoted after *RECQL4* overexpression (Figure 4a). In addition, cell senescence was heightened after inhibiting *RECQL4* but retarded after overexpression of *RECQL4* (Figure 4b). Taken together, *RECQL4* relieved both cell cycle arrest and cell senescence.

RECQL4 activated NF-κB and ERK signaling pathways

We probed whether *RECQL4* modulated the NF-κB and ERK signaling pathways. Western blot analysis illuminated that the levels of phospho(p)-p65/p65 and p-ERK/ERK were reduced after *RECQL4* repression but enhanced after *RECQL4* overexpression (Figure 5). This data revealed that *RECQL4* activated NF-κB and ERK signaling pathways in LUAC.

Cortex Mori extract exhibited antitumor effects in LUAC

It was demonstrated that CM (5 mg/mL) treatment reduced cell viability (Figure 6a). In addition, cell migration and invasion were weakened after CM (5 mg/mL) treatment (Figures 6b–6e). The E-cadherin protein expression was increased, but the N-cadherin, snail, slug and vimentin protein expressions were decreased

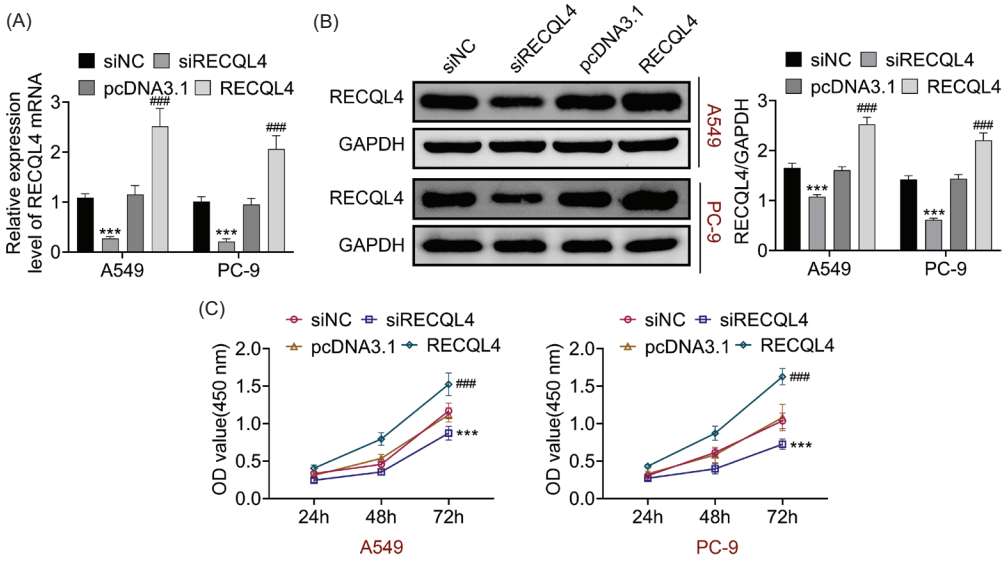

Figure 2 ***RECQL4*** **enhanced cell proliferation. (A,B) The knockdown and overexpression efficiency of** ***RECQL4*** **in A549 and PC-9 cells were verified through RT-qPCR and Western blot analysis. (C) The cell proliferation was measured after silencing or up-regulating** ***RECQL4*** **through CCK-8 assay. ****P* **< 0.05, ******P* **< 0.001 vs siNC; ###***P* **< 0.001 vs pcDNA3.1. NC: negative control.**

after CM (5 mg/mL) treatment (Figure 6f). Besides, the S phase of cell cycle was retarded after CM (5 mg/mL) treatment (Figure 6g). In general, CM extract exhibited antitumor effects in LUAC.

Cortex Mori extract blocked *RECQL4*-induced NF-κB and ERK signaling pathways

The *RECQL4* protein expression was reduced after CM (5 mg/mL) treatment (Figure 7a). In addition, the levels of both p-p65/p65 and p-ERK/ERK were decreased following CM (5 mg/mL) treatment (Figure 7b). These findings indicated that CM extract blocked *RECQL4*-induced NF-κB and ERK signaling pathways.

Discussion

RECQL4 has been shown to exhibit higher expressions and act as a facilitator in many types of cancers. For example, *RECQL4* aggravates resistance to oxaliplatin by modulating PI3K/AKT signaling pathway in colon adenocarcinoma (Zhou *et al*., 2021)]. In hepatocellular carcinoma, the increased *RECQL4* expression results in poor prognosis (Li *et al*., 2018a). Moreover, suppression of *RECQL4* weakens cell invasion ability in metastatic prostate cancer (Su *et al*., 2010). In the present work, it was discovered that the higher expression of *RECQL4* existed in LUAC tissues and cell lines.

In all, 90% cancer-related deaths are caused by metastasis, but this process is one of the most unintelligible pathological processes in development of cancers (Suhail *et al*., 2019). The development of novel treatment strategies for key factors driving metastasis is a very challenging objective for researchers (Chaffer and Weinberg, 2011).

In embryogenesis, the EMT process occurs in embryonic cells, resulting in epithelial cells to move to distant places (Nieto, 2013; Thiery *et al*., 2009). Once the migrated embryonic cells reach their destination, they undergo an opposite process, that is, the transformation from mesenchymal cells to epithelial cells (MET), so as to proliferate and differentiate into diverse organs (Nieto, 2013). Similarly, cancer cells also own metastatic process (Zhang and Weinberg, 2018). EMT progression provides cancerous epithelial cells the migration and invasion abilities, allowing them to enter the mesenchymal-like state (Bischoff, 2019). This change results in metastasis and colonization of primary tumor in distal organs, and forms secondary tumors (Piera-Velazquez and Jimenez, 2019) In addition, these cancer cells after EMT progression are discovered as frequently resistant to radiotherapy/chemotherapy drugs and are accompanied by tumor recurrence and re-metastasis (Du and Shim, 2016; Pastushenko and Blanpain, 2019). Understanding the molecular regulation mechanisms of EMT progression could provide novel targets and options for effective cancer treatment. In our study, through functional experiments, we found that *RECQL4* facilitated cell proliferation and EMT progression. In addition, through flow cytometry and SA-β-gal staining assays, *RECQL4* had been proved to relieve cell cycle arrest and cell senescence.

The NF-κB and ERK signaling pathways are highly active in diversified cancers, and aggravate tumor growth,

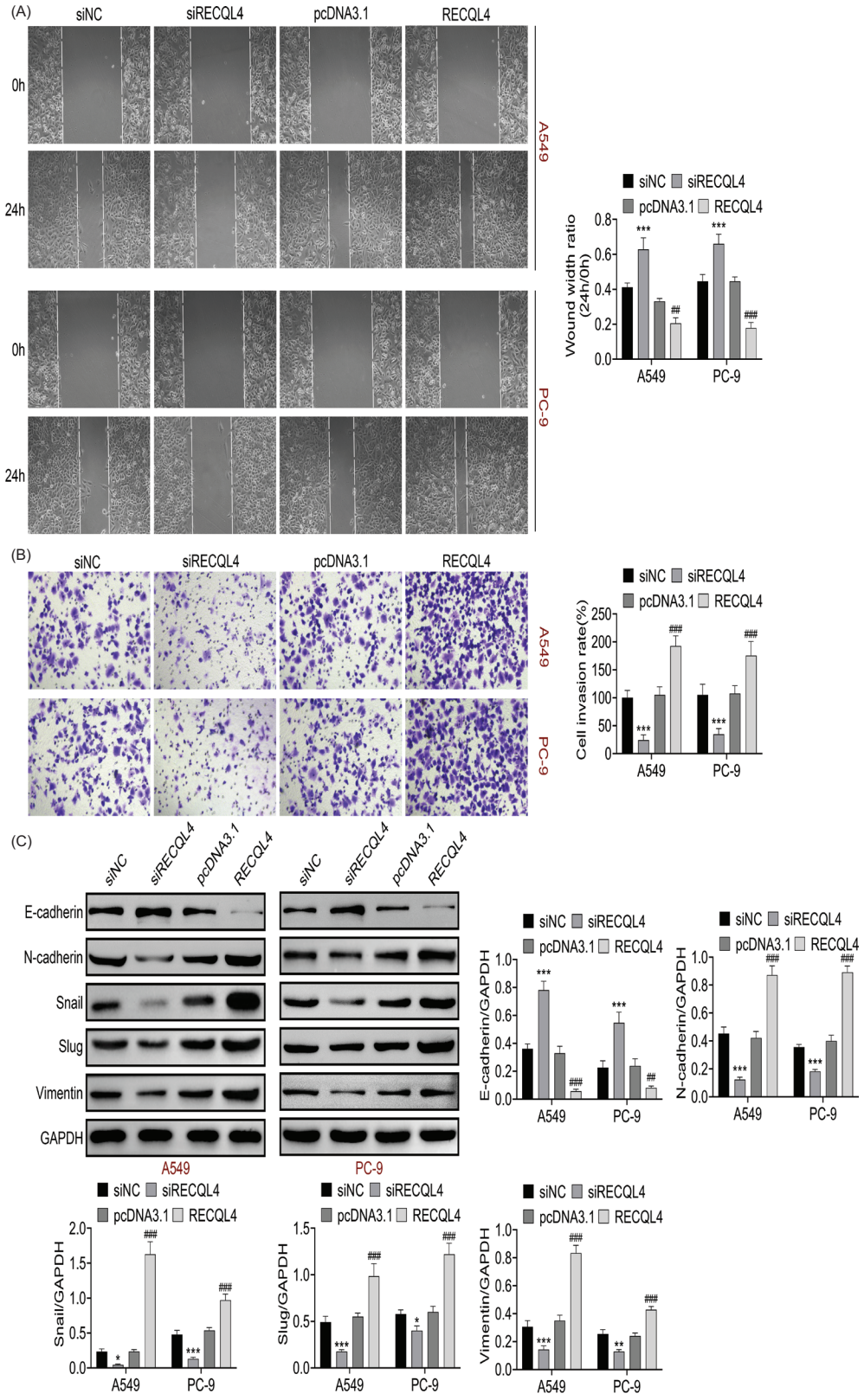

Figure 3 ***RECQL4*** **facilitated cell migration and invasion as well as EMT progression. (A) The migration ability was measured after inhibiting or overexpressing** ***RECQL4*** **through wound healing assay. (B) The invasion ability was discovered after** ***RECQL4*** **knockdown or overexpression through transwell assay. (C) The EMT progression-related proteins (E-cadherin, N-cadherin, snail, slug, and vimentin) were inspected by Western blot analysis. ****P* **< 0.05, *****P* **< 0.01, ******P* **< 0.001 vs siNC; ##***P* **< 0.01, ###***P* **< 0.001 vs pcDNA3.1. NC: negative control.**

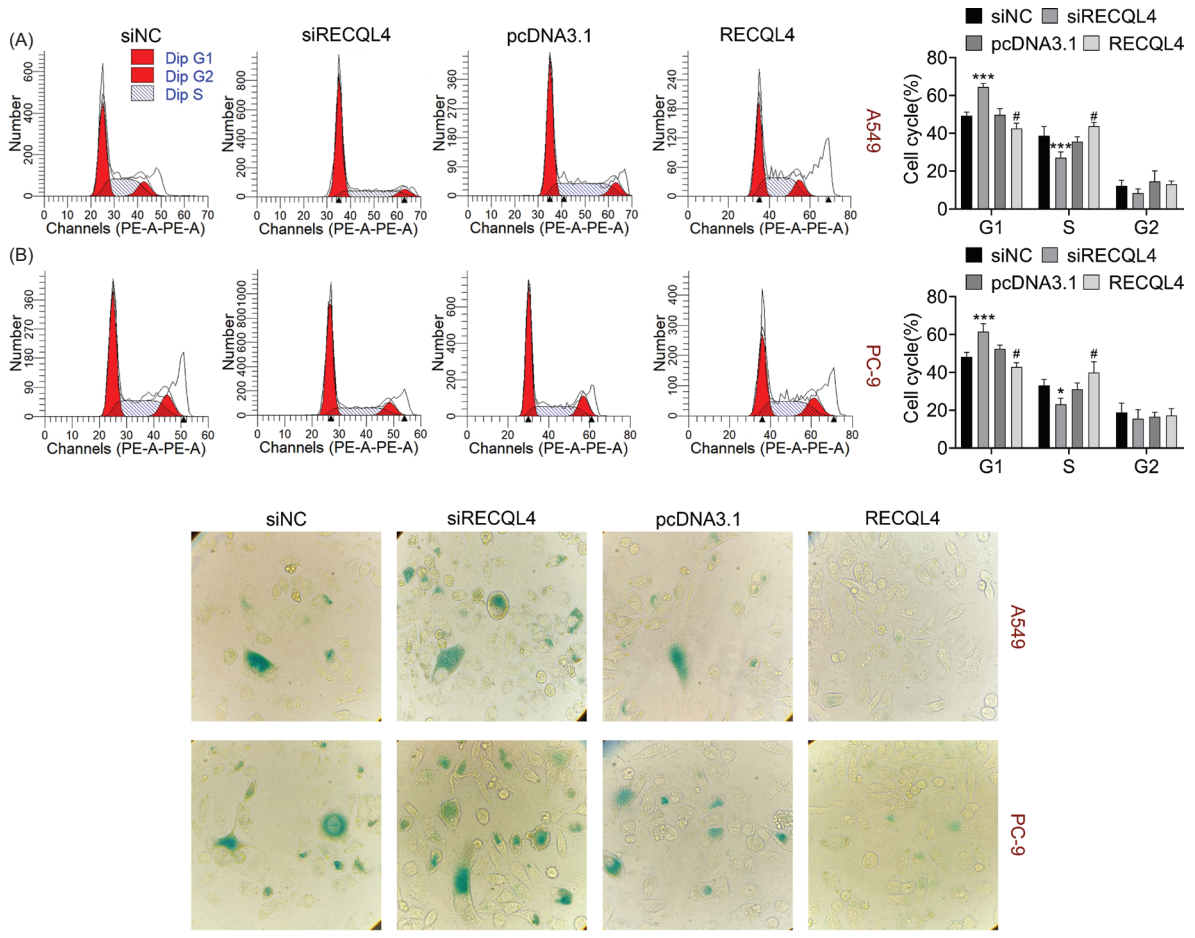

Figure 4 ***RECQL4*** **relieved cell cycle arrest and cell senescence. (A) The cell cycle was evaluated after** ***RECQL4*** **suppression or overexpression through flow cytometry. (B) The cell senescence was confirmed after** ***RECQL4*** **down-regulation or up-regulation through SA-β-gal staining. ****P* **< 0.05, ******P* **< 0.001 vs siNC; #***P* **< 0.05 vs pcDNA3.1. NC: negative control.**

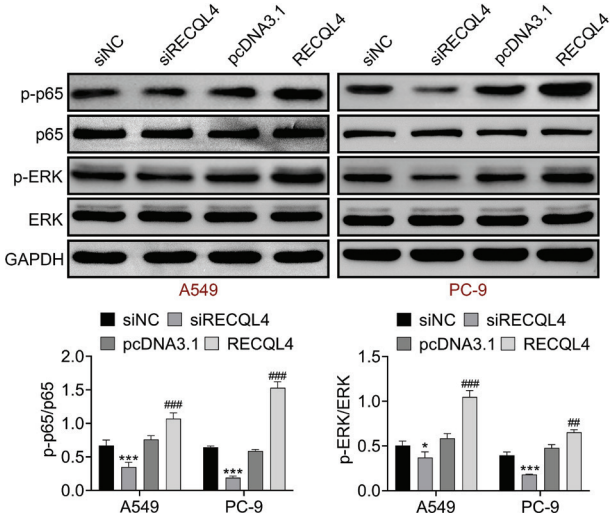

Figure 5 ***RECQL4*** **activated NF-κB and ERK signaling pathways. The protein expressions of p-p65, p65, p-ERK, and ERK were examined after silencing or overexpressing** ***RECQL4*** **through Western blot analysis. ****P* **< 0.05, ******P* **< 0.001 vs siNC; ##***P* **< 0.01, ###***P* **< 0.001 vs pcDNA3.1. NC: negative control.**

metastasis, and EMT progression. For instance, BMP9 modulates the MAPK/ERK and NF-κB signaling pathways to suppress the proliferation and migration in LUAC (Wang *et al*., 2016). Osteopontin affects the PI3K, AKT, ERK, and NF-κB pathways to exacerbate cell migration in lung cancer (Fong *et al*., 2009). Perfluorooctanoic acid (PFOA) regulates the ERK/NF-κB/MMP-2/-9 pathways to induce cell metastasis in ovarian cancer (Li *et al*., 2018b). Puerarin retards the NF-κB and ERK signaling pathways to reduce cell migration and adhesion in LPS-mediated breast cancer (Liu *et al*., 2017). In our study, it was revealed that *RECQL4* activated NF-κB and ERK signaling pathways by enhancing p-p65/p65 and p-ERK/ERK levels in LUAC.

Cortex Mori extract has been investigated to affect cellular progresses to regulate different diseases. For example, CM extract improves insulin resistance and nonalcoholic fatty liver disease in high-fat diet-/streptozotocin-stimulated type 2 diabetes (Ma *et al*., 2018). In addition, CM extract suppresses microtubule assembly to trigger cancer cell

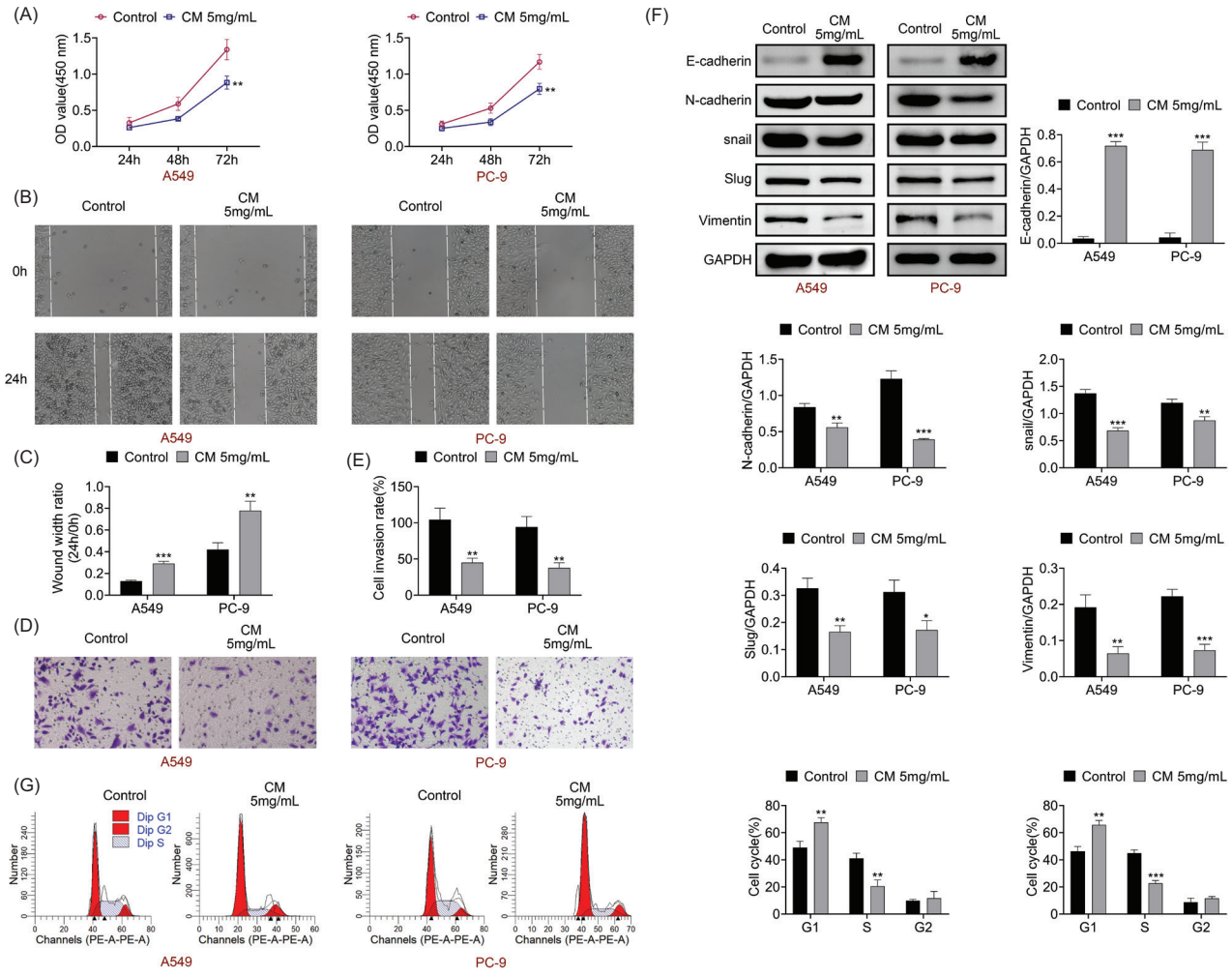

Figure 6 Cortex Mori extract exhibited antitumor effects in LUAC. (A) Cell viability was measured after CM (5 mg/mL) treatment through CCK-8 assay. (B,C) Cell migration was tested after CM (5 mg/mL) treatment through wound healing assay. (D,E) Cell invasion was examined after CM (5 mg/mL) treatment through Transwell assay. (F) The protein expressions of E-cadherin, N-cadherin, snail, slug and vimentin were confirmed after CM (5 mg/mL) treatment through Western blot analysis. (G) The cell cycle was detected after CM (5 mg/mL) treatment through flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001 vs control.

apoptosis (Nam *et al*., 2002). Besides, CM extract retards PI3K/AKT signaling to enhance apoptosis and reduce tumor invasion in melanoma (Hu *et al*., 2022). However, the detailed functions of *RECQL4* and CM extract, as well as their regulatory mechanisms, in LUAC have not been illustrated. In this study, it was discovered that CM extract exhibited antitumor effects in LUAC, and blocked *RECQL4*-induced NF-κB and ERK signaling pathways.

In short, it has been discovered for the first time that CM extract inhibited migration and invasion of LUAC cells by blocking *RECQL4*-induced NF-κB and ERK signaling pathways. The discovery could help clarify the functioning of *RECQL4* in improving the CM treatment of LUAC.

Nevertheless, this study also had some limitations concerning the effects of *RECQL4* on CM treatment in LUAC progression, such as the functions of *RECQL4* and CM extract in LUAC clinical samples, animal models and other cellular progression are not involved. In the future, more experiments are required to probe the other functions of CM extract, and its regulatory mechanism on *RECQL4* in LUAC progression.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors stated that there was no conflict of interest to disclose.

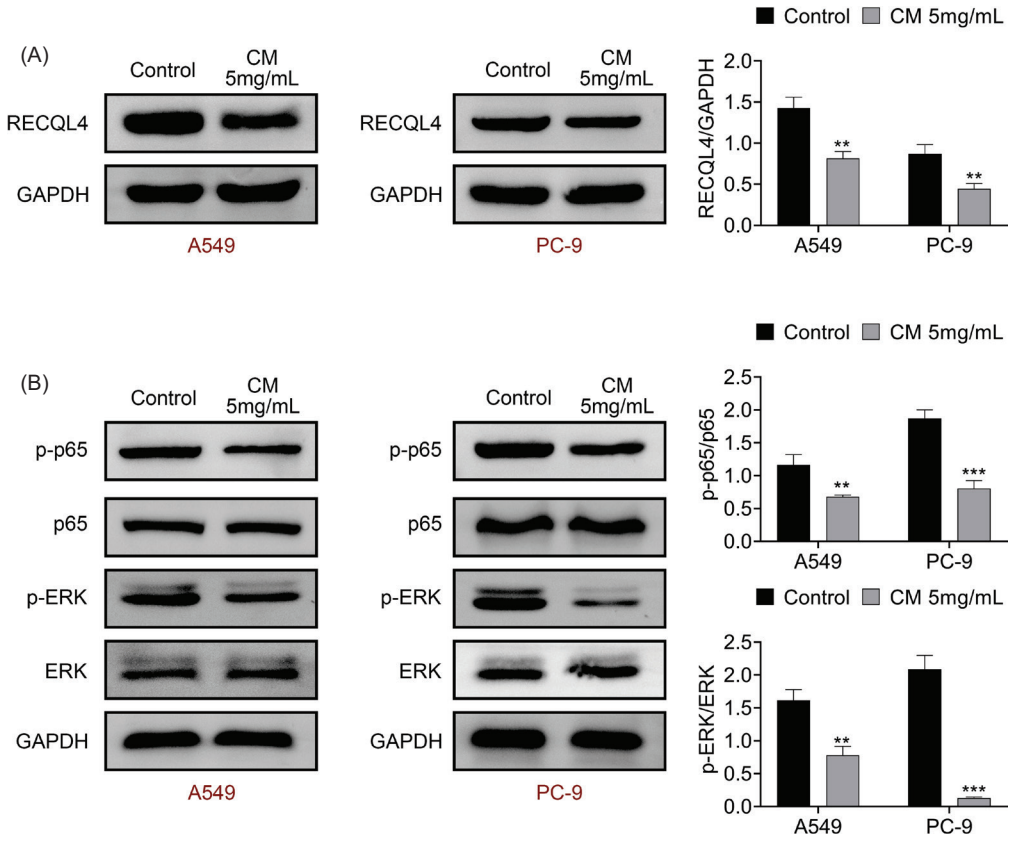

Figure 7 Cortex Mori extract blocked *RECQL4*-induced NF-κB and ERK signaling pathways. (A) The protein expression of *RECQL4* was tested after CM (5 mg/mL) treatment through Western blot analysis. (B) The protein expressions of p-p65, p65, p-ERK, and ERK were examined after CM (5 mg/mL) treatment through Western blot analysis. **$P < 0.01$, ***$P < 0.001$ vs control.

Ethics approval

Not applicable.

Author Contributions

Qin Li and Enyao Wei designed the study, and supervised data collection. Wenbin Zhang analyzed and interpreted the data. Feng Zhang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

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