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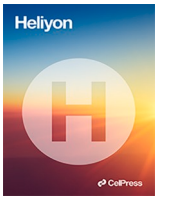

Research article

The COL7A1/PI3K/AKT axis regulates the progression of cholangiocarcinoma

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ABSTRACT

Background: The role and molecular mechanisms of collagen type VII (COL7A1) in cholangiocarcinoma (CCA) remain unknown.
Methods: We analyzed the expression of COL7A1 in CCA and its relationship with patient prognosis using bioinformatic techniques. Expression levels of COL7A1 in CCA cells and tissues were detected using reverse transcription-quantitative PCR, western blotting, and immunohistochemistry. The effects of COL7A1 expression on the proliferation, migration, and invasion of CCA cells were assessed using CCK-8, colony formation, and Transwell assays. Bioinformatics and luciferase reporter gene assays were performed to examine the binding of KLF4 to COL7A1, and cytological experiments further verified the role of KLF4 in regulating the CCA phenotype through COL7A1. Xenograft mouse models were established to investigate the effects of COL7A1 on CCA tumor growth *in vivo*.
Results: CCA tissues exhibited higher COL7A1 expression than normal bile duct tissues. There was no significant correlation between high or low COL7A1 expression and the survival time of patients with CCA. COL7A1 knockdown inhibited CCA cell proliferation, migration, and invasion. Furthermore, COL7A1 knockdown suppressed the activation of the PI3K/AKT signaling pathway. KLF4 can bind to COL7A1 and regulate COL7A1 expression, which in turn regulates the PI3K/AKT signaling pathway and impacts the proliferation and metastasis of CCA cells.
Conclusion: Our findings suggest that KLF4 regulates CCA cell proliferation, migration, and invasion via the COL7A1/PI3K/AKT axis.

1. Introduction

Cholangiocarcinoma (CCA) is a type of malignant tumor that originates from the epithelial cells of the bile ducts, and its incidence is increasing annually worldwide. Owing to its insidious onset and inconspicuous symptoms during the early stages, most patients are

Abbreviations: COL7A1, collagen type VII; CCA, cholangiocarcinoma; IHC, immunohistochemistry; IF, immunofluorescence; TCGA, The Cancer Genome Atlas; KLF4, Krüppel-like4.

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diagnosed in the middle or late stages [1,2]. The incidence of this type of tumor is increasing worldwide. Depending on their location, CCAs can be divided into intrahepatic and extrahepatic CCA [3]. Currently, radical surgical resection is the only available treatment option for bile duct cancers. Radical surgical resection is the only effective treatment for CCA. However, this was limited to a small number of patients diagnosed at an early stage of the disease. Most patients already present with lymphatic, intrahepatic, or distant organ metastases at the time of diagnosis, which leads to a limited 5-year survival rate of 20–30 % [4]. Exploring the molecular mechanisms underlying CCA cell proliferation and metastasis will provide a theoretical basis for identifying crucial tumor targets and guiding clinical treatment.

Krüppel-like4 (KLF4) is an evolutionarily conserved member of the zinc-finger transcription factor family that was first identified in murine differentiated epithelium, colon, and small intestine [5,6]. KLF4 is reportedly involved in the regulation of cell proliferation and differentiation and plays a vital role in cell growth [7]. KLF4 plays an essential role in tumorigenesis and development and exerts a bidirectional regulatory role owing to its unique structure; that is, it can be expressed in different tumors to promote or inhibit simultaneously, such as breast, gastric, and intestinal tumors [8–10]. Bidirectional regulation of tumorigenesis is also an aspect of KLF4 that distinguishes it from most transcription factors, possibly because KLF4 transcriptional repression or activation domains differ in different tumor types [11].

COL7A1 encodes type VII collagen, composed of three identical a-chains, and is 145 kDa long. COL7A1 is a key component of anchoring collagen fibers and is closely related to the attachment structure within the dense layer of the basement membrane. COL7A1 is mainly responsible for epidermal-dermal adhesion in the skin [12,13]. Mutations in the COL7A1 gene are frequently observed in invasive cutaneous squamous cell carcinoma, and deletion of type VII collagen has been found to promote the invasive metastasis of cutaneous squamous cell carcinoma, regulate cellular differentiation, and promote the epithelial-mesenchymal transition of cells [14, 15]. COL7A1 is highly expressed in cutaneous squamous cell carcinoma cells, upregulates the expression of signaling molecules such as PI3K and MAPK, and promotes cell migration and invasive capacity [16]. These findings suggest that COL7A1 plays a vital role in tumor development, especially in invasion and metastasis.

In the current study, we predicted the mutual binding effects of KLF4 and COL7A1 using bioinformatics techniques and analyzed the downstream pathways using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Cytological experiments were performed to elucidate molecular mechanisms of KLF4 and COL7A1 in CCA.

2. Materials and methods

2.1. Cell culture

Four CCA cell lines (KMBC, QBC939, HCC9810, and RBE) and one immortalized biliary epithelial cell line (HIBEPIC) were purchased from the cell bank of the Chinese Academy of Sciences. The cells were cultured in RPMI 1640 serum-free medium or DMEM high-glucose medium supplemented with 1 % penicillin/streptomycin and 10 % fetal bovine serum (FBS; BI, C04001-050). Cells were cultured in an incubator set at 37 °C with 5 % CO2.

2.2. Stabilized CCA cell line construction

Three knockdown plasmids of COL7A1 (Shanghai General, Shanghai, China) with the best results were selected for lentiviral packaging. The CCA cell line, KMBC, was inoculated into 6-well plates and cultured overnight. When the cell density reached ~80 %, the lentiviral COL7A1 knockdown vector and empty lentiviral vector were added, and the cells were cultured for an additional 24 h. The virus-containing medium was replaced with a fresh medium. After another 24 h of culture, puromycin was used to screen stable KMBC cells for lentiviral transfection.

2.3. Transient transfection of CCA cells

CCA cells in the logarithmic growth phase were seeded in six-well plates. When cells reached ~80 % confluency, the small interfering RNA (siRNA) fragment (Shanghai General, China) was diluted as required, the old medium in the six-well plate was removed, and opti-DMEM medium was added, which was mixed with the siRNA according to the instructions of the lip3000 transfection reagent (Thermo Fisher, USA, L3000075). The cells were maintained at 25 °C for 15–20 min to form transfection complexes in the six-well plates. After 6–8 h, the medium was replaced with a fresh medium. Follow-up experiments were performed after 48 h of incubation.

2.4. Reverse transcription-quantitative PCR (RT-qPCR) to detect gene expression

Cell samples were collected from each group, and RNA was extracted from each sample using the TRIzol reagent (Biosharp, China, BS258A) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the Reverse Transcription Reagent Kit (Thermo Fisher, USA, K1691). RNA was extracted using TB Green®Premix Ex Taq™II (Tli RNaseH Plus) (Takara Bio, Japan, RR820A) on a StepOnePlus PCR system (Applied Biosystem, USA) for RT-qPCR. GADPH was used as an internal reference and calculated as 2–ΔΔCt.

2.5. CCK-8 assay to detect cell proliferation

Cell viability was assessed using a CCK-8 assay (Biosharp, China; BS350A). Briefly, cells were inoculated in 96-well plates (1 × 103 cells per well) and incubated with RPMI 1640 medium containing 10 % FBS for 24 h. Cells were then incubated with CCK-8 solution for 2 h (final concentration: 10 %). Absorbance was measured at 450 nm using a Synergy HT Multi-Mode Microtiter Plate Instrument (BioTek, USA) every 24 h for five consecutive days.

2.6. Clone formation experiments

In brief, cells were inoculated into 6-well plates (2 × 103 cells/well) and cultured for approximately two weeks to form visible colonies. The cells were fixed with 4 % paraformaldehyde (Biosharp, China, BL539A) and stained with 0.1 % crystal violet (Beyotime, China; C0121-100 mL). The colonies were photographed and quantitatively analyzed using the ImageJ version 1.35 k (National Institutes of Health, Bethesda, MD, USA).

2.7. Transwell experiment

A transwell system (Corning, USA, CLS3422) containing 8.0 μm wells was used to assay the migration and invasion capacity of cells. Cells in the logarithmic growth phase were serum-starved overnight, and for cell invasion assays, transwell inserts were coated with 50 μl of a mixture of serum-free RPMI 1640 and Matrigel (1:8) and cured at 37 °C for 4 h. The upper chamber was inoculated with 100 μl of serum-free RPMI 1640 cells and left to stand for 20 min, while the lower chamber was supplemented with 600 μl of complete RPMI 1640 medium. For the cell migration assay, cells were implanted in the upper chamber and incubated for 24 h. Cells that migrated into the lower chamber were washed three times with phosphate-buffered saline, fixed with 4 % paraformaldehyde for 15 min, and stained with 0.1 % crystal violet for 30 min at 25 °C. The number of cells that had migrated through the wells or invaded the matrix was counted. Cells were counted in five representative fields of view for each membrane layer.

2.8. Immunohistochemical (IHC) and immunofluorescence (IF)

The experimental step for IHC scoring was performed as described previously [17]. Paraffin sections of the CCA and adjacent non-tumor tissues were deparaffinized and hydrated. Endogenous peroxidase activity was blocked using 3 % hydrogen peroxide. Slides were incubated overnight with a primary antibody against COL7A1 (1:200) and then with horseradish peroxidase-conjugated secondary antibody for 1 h at 25 °C. Images were captured and analyzed for expression.

Cells in 6-well plates were fixed with 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100, and blocked with 5 % bovine serum albumin. The samples were incubated overnight with the primary antibody, followed by 1 h at 25 °C incubation with a coralite594-conjugated fluorescent secondary antibody. Cells were stained with DAPI and imaged under an inverted fluorescence microscope.

2.9. Apoptosis assay

A cell cycle and apoptosis assay kit (C1052; Biyun Tian, China) was used for cell cycle and apoptosis analysis using the classical propidium iodide staining method. Different groups of cells were resuspended at a density of 5×10^5 cells/ml, incubated with PI, and analyzed by flow cytometry (Agilent Technology, USA).

2.10. Dual fluorescent reporter genistein assay

Cells in the logarithmic growth phase were selected and spread onto plates. Transfection was performed when approximately 80 % of cells were fused. The modified COL7A1 reporter gene plasmid, KLF4 overexpression plasmid, and positive control plasmid were co-transfected into the cells. After 48 h, the medium was discarded. The cells were washed with phosphate buffer once, and the cell lysate was added to the cell surface to cover it completely; the reaction was performed for 30 min. Firefly/sea kidney luciferase substrate was collected by centrifugation, diluted according to the manufacturer's instructions, prepared into appropriate dilutions, and set aside. Then, 100 μl/well of the cell lysate was added according to the group in triplicate. Firefly luciferase reaction solution was added to each well (10 μl/well), and the activity of the corresponding luciferase enzyme was detected. Finally, Sea Kidney Luciferase Reaction Solution (10 μl/well) was added, and the activity of the corresponding luciferase enzyme was detected.

2.11. Xenograft mouse models

In brief, KMBC cells (1×10^7) were injected into the subcutaneous tissues of 4-week-old female BALB/c-nu thymic nude mice (Hangzhou Resource Laboratory Animal Science and Technology Co., Ltd., China; n = 5/each group). Tumor growth was observed every 3–4 days, and the tumor volume was recorded.

2.12. Bioinformatics analysis

RNA datasets for CCA and normal tissues were downloaded from The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/), and the differential expression of COL7A1 in CCA, gene set enrichment analysis (GSEA), and survival information were analyzed using the R language. To analyze the expression of COL7A1 in each cancer, we examined COL7A1 differential expression in CCA using the TIMER2.0 website. The downstream pathways of COL7A1 were searched using GO and KEGG analyses, and data were analyzed using the SangerBox platform (https://sangerbox.com).

2.13. Statistical analysis

The student's t-test was used to compare the means of two groups of data. One-way ANOVA was used to compare the means of three or more groups. The relationship between gene expression and patient characteristics was tested using the chi-squared test. All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

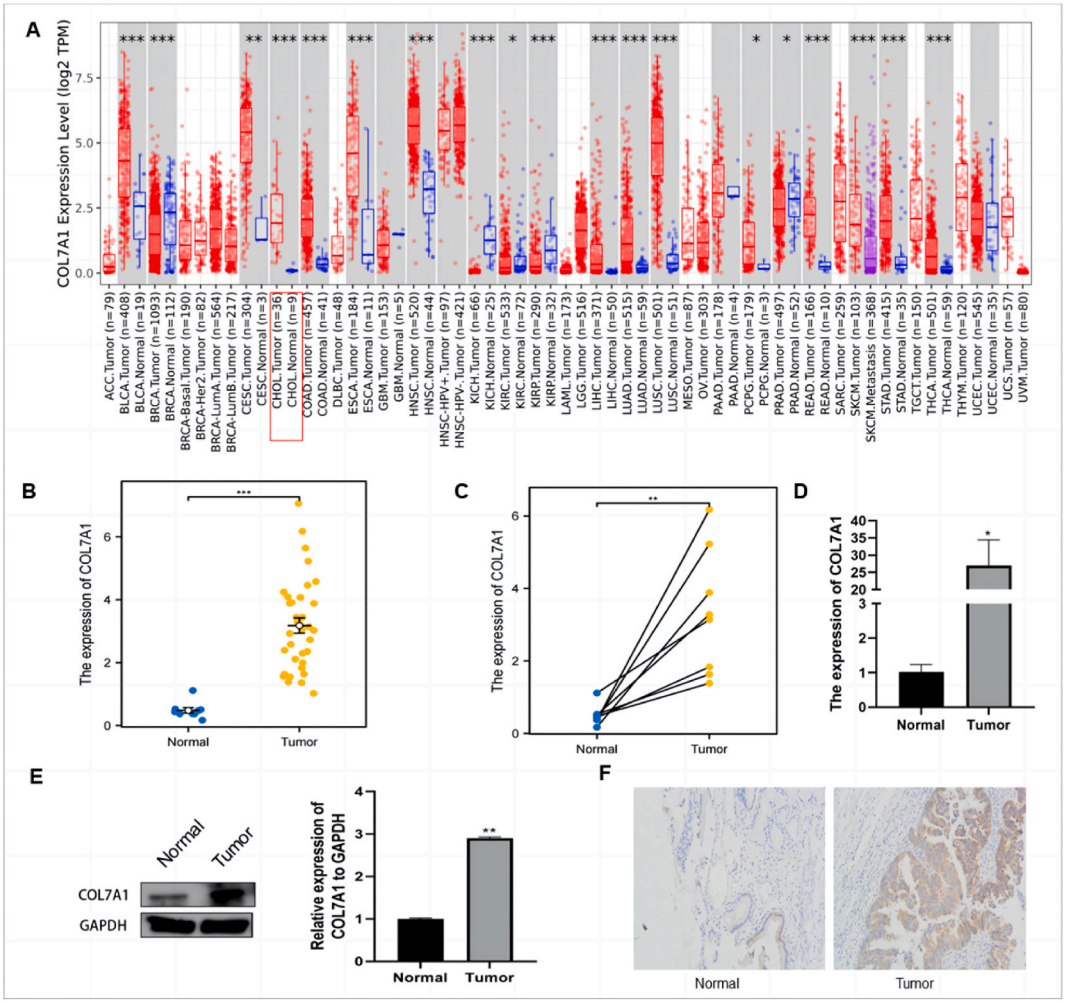

Fig. 1. COL7A1 is highly expressed in cholangiocarcinoma (A) Pan-cancer analysis showed that COL7A1 was highly expressed in cholangiocarcinoma. (B,C) Cohort and pairwise analyses of COL7A1 based on transcriptomic data in TCGA. (D–F) RT-qPCR, Western blot analysis, and immunohistochemical detection of COL7A1 expression in CCA tumor tissues and normal bile duct tissues (The uncropped images are referenced in original blot: Fig. 1E).*p < 0.05, **p < 0.01, ***p < 0.001. CCA, cholangiocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; TCGA, The Cancer Genome Atlas.

3. Results

3.1. COL7A1 is highly expressed in CCA tissues

We analyzed COL7A1 expression in various cancers using the TIMER2 database. The results showed that COL7A1 was highly expressed in several cancers, particularly CCA (Fig. 1A). Notably, COL7A1 expression was not associated with overall survival, progression-free intervals, disease-specific survival, and disease-free survival in patients (Supplementary Image 1). COL7A1 was highly expressed in CCA in TCGA database (Fig. 1B and C). Detection of CCA samples and normal tissues using RT-qPCR, western blotting, and IHC revealed that COL7A1 was highly expressed in tumor tissues (Fig. 1D–F).

3.2. Knockdown of COL7A1 inhibits proliferation, migration, and invasion of CCA cells

We examined COL7A1 expression in four strains of CCA cells and one strain of bile duct epithelial cells using RT-qPCR and western blotting analyses (Supplementary Figs. 2A and B). Based on these experimental results, KMBC and QBC939 were selected for subsequent experiments, and CCK-8 and clone formation assays were performed. In CCA cells, COL7A1 knockdown inhibited the proliferative (Fig. 2A and B), migration, and invasion abilities (Fig. 2C–E). Flow cytometry revealed that COL7A1 knockdown increased the apoptotic rate of cells (Fig. 2F). Collectively, these findings indicated that the knockdown of COL7A1 could inhibit the proliferation, migration, and invasion of CCA cells.

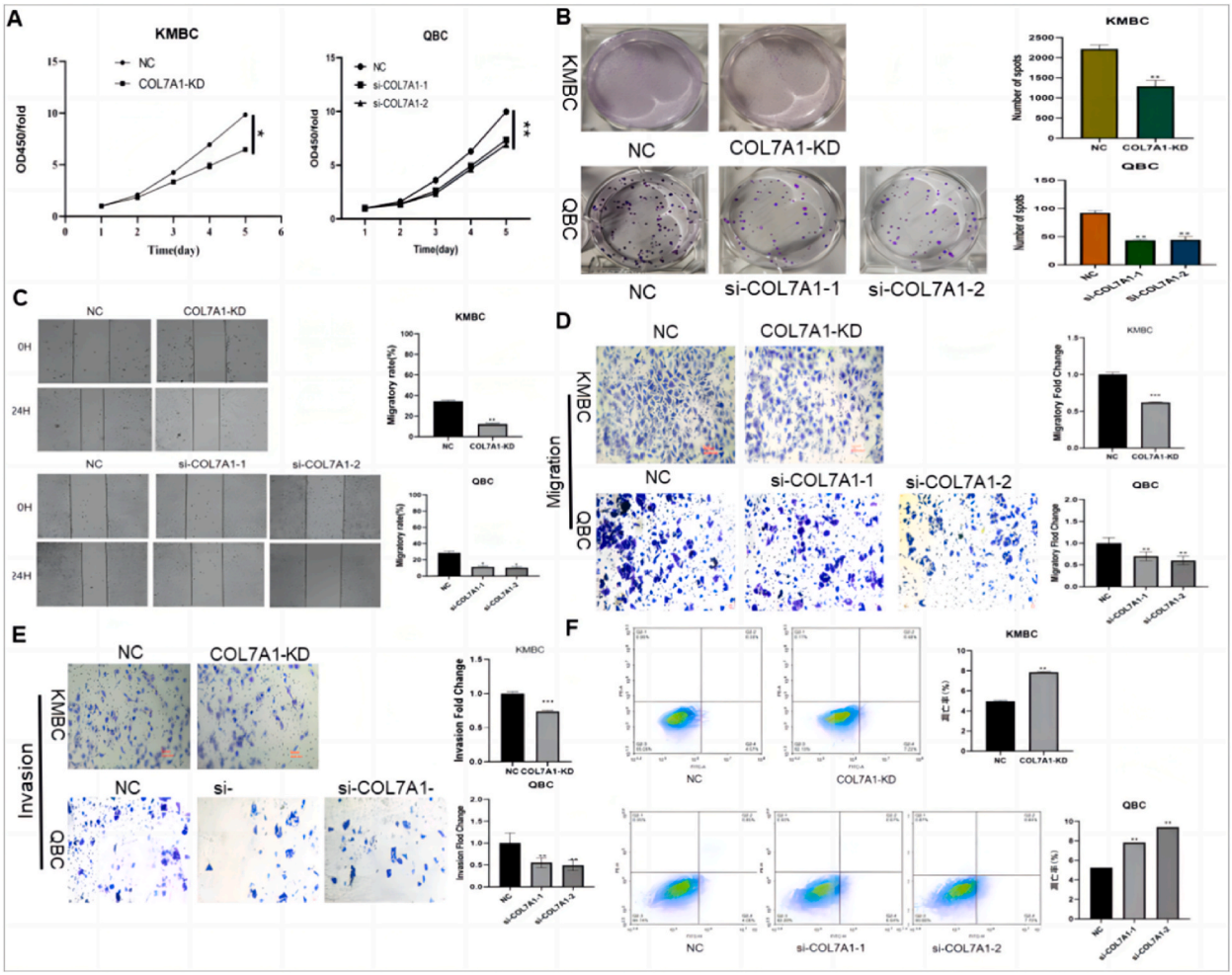

Fig. 2. Knockdown of COL7A1 inhibits proliferation, migration, and invasion of CCA cells. (A–B) CCK8 and clone formation assays to detect the proliferative ability of KMBC and QBC939 cells; (C) scratch assay to detect the migratory ability of KMBC and QBC939 cells; (D–E) Transwell assay to detect the migratory and invasive ability of KMBC and QBC939. (F) Flow cytometry assay to detect apoptosis of KMBC and QBC939 cells. Each set of experiments was performed in triplicate.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CCA, cholangiocarcinoma.

3.3. COL7A1 regulates the PI3K/AKT signaling pathway

CCA data in TCGA were processed using R language, and a heat map of differential gene expression was plotted according to the level of COL7A1 expression (Fig. 3A). Using the SangerBox data platform, GO analysis revealed that the COL7A1 function was related to lipid localization and wound healing (Fig. 3B), whereas KEGG enrichment analysis showed that the function of COL7A1 was related to the PI3K/AKT signaling pathway (Fig. 3C). GSEA revealed that COL7A1 was associated with adipogenesis, bile acid metabolism, and oxidative phosphorylation and negatively correlated with epithelial-mesenchymal transition and transforming growth factor β signaling (Fig. 3D and E). Knockdown of COL7A1 reduced protein levels of p-PI3K and p-AKT, as evidenced by western blotting analysis (Fig. 3F–H). Therefore, we speculated that COL7A1 can regulate the phosphorylation of the PI3K/AKT signaling pathway.

3.4. KLF4 regulates COL7A1 expression

Based on predictions from the NCBI and UCSC databases, KLF4 was identified as a potential transcription factor upstream of COL7A1 (Fig. S3). TCGA dataset showed that KLF4 was highly expressed in CCA (Fig. 4A). We found that KLF4 was weakly expressed in KMBC cells and highly expressed in QBC939 cells (Fig. 4B–C). We constructed and validated QBC939 by knocking down KLF4 in the KMBC cell line and overexpressing KLF4 (Fig. 4D). To further validate the correlation between KLF4 and COL7A1, we constructed wild-type and mutant groups of COL7A1 using a double luciferase assay. Herein, the fluorescence intensity of the mutant group was significantly lower than that of the wild-type group (Fig. 4E–F). After KLF4 knockdown, the fluorescence intensity of COL7A1 was

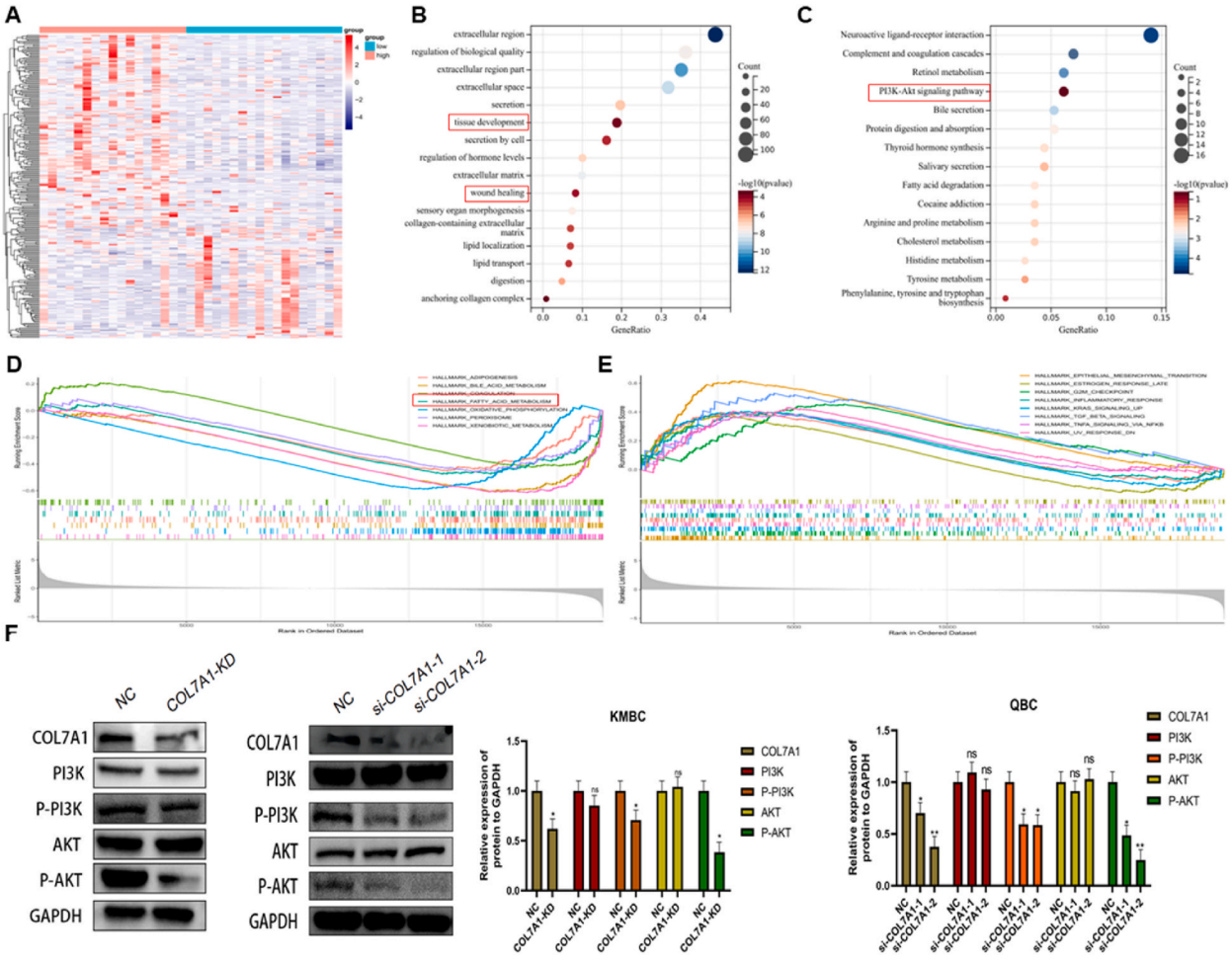

Fig. 3. COL7A1 regulates phosphorylation of the PI3K/AKT signaling pathway (A) Heatmap of differential genes in TCGA transcriptome data. (B) Enrichment of differential genes for cellular functions in GO. (C) Enrichment analysis of differential gene signaling pathways in KEGG. (D–E) Functional enrichment analysis of the differential genes on GSEA showed that COL7A1 expression was associated with fatty acid metabolism. (F–H) Western blotting analysis to detect protein expression in the PI3K/AKT signaling pathway following COL7A1 knockdown (The uncropped images are referenced in original blot: Fig. 3F). *p < 0.05, **p < 0.01, ***p < 0.001. GO, Gene Ontology; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

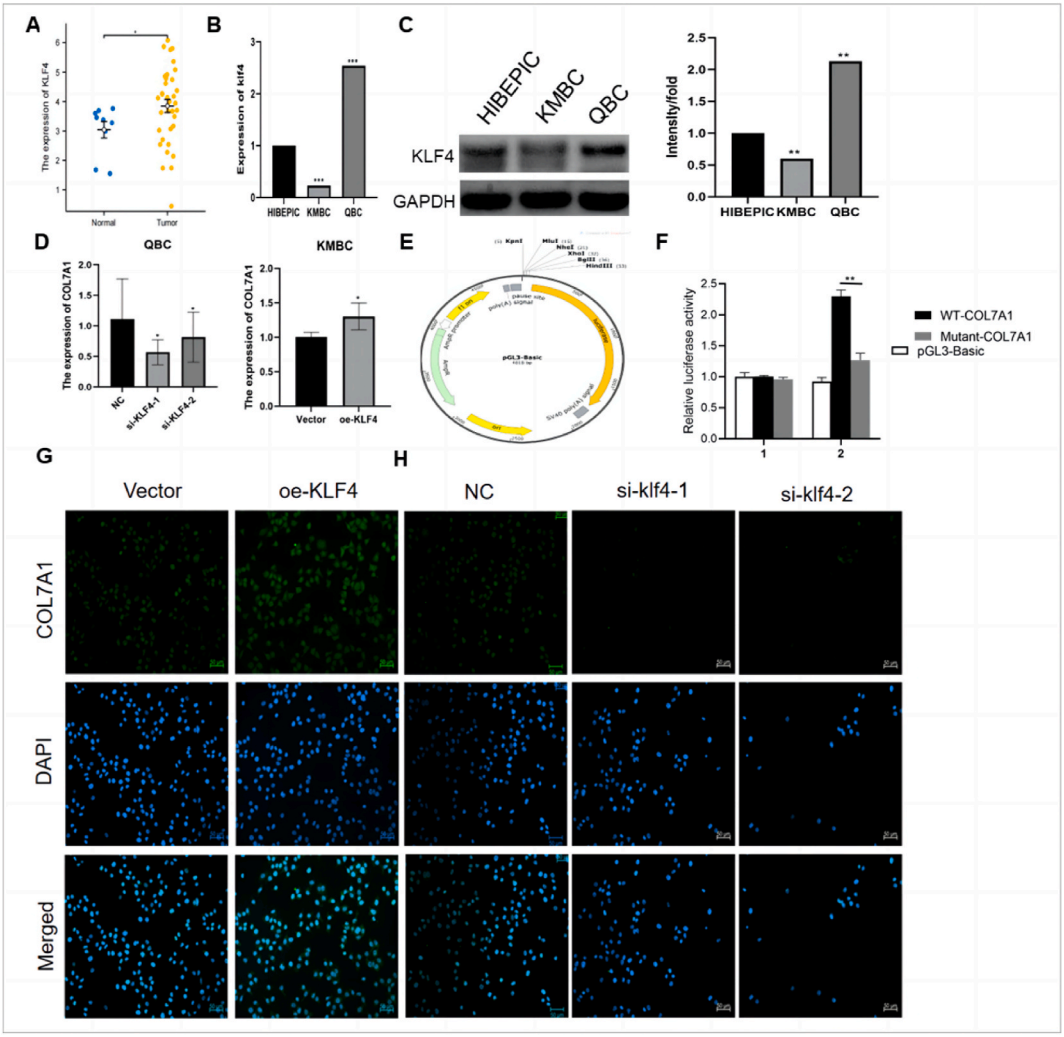

Fig. 4. KLF4 regulates COL7A1 expression (A) TCGA dataset analysis of KLF4 expression in CCA. (B) RT-qPCR was performed to detect the mRNA level of KLF4 in KMBC and QBC939 cells. (C) Western blotting was performed to detect the protein level of KLF4 in KMBC and QBC939 cells (The uncropped images are referenced in original blot: Fig. 4C). (D) RT-qPCR was performed to detect the expression level of COL7A1 after KLF4 knockdown. (E) Mutation sites in COL7A1. (F) Dual-luciferase assay was used to detect the correlation between KLF4 and COL7A1 expression. (G–H) Immunofluorescence detection of COL7A1 expression after overexpression and knockdown KLF4. Each set of experiments was performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CCA, cholangiocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; TCGA, The Cancer Genome Atlas; WT, wild-type; Scale: 1:200.

considerably reduced, as detected by IF detection (Fig. 4G–H). These results suggested that KLF4 regulates the expression of COL7A1.

3.5. *Knockdown of KLF4 inhibits proliferation, migration, and invasion of CCA cells*

Knockdown of KLF4 inhibited the proliferation of QBC939 cells, and overexpression of KLF4 promoted the proliferative ability of KMBC cells, as demonstrated by CCK-8 and clone formation assays (Fig. 5A and B). The migration and invasion ability of CCA cells could be affected by altering the expression of KLF4 (Fig. 5C–E). Further validation of the effect of KLF4 on apoptosis in CCA cells revealed that overexpression of KLF4 suppressed apoptosis in CCA cells, while inhibition of KLF4 promoted cellular apoptosis (Fig. 5F). Although knockdown or overexpression of KLF4 did not induce notable changes in the expression of PI3K and AKT, the expression of p-PI3K and p-AKT was markedly altered. KLF4 knockdown inhibited the phosphorylation of the PI3K/AKT signaling pathway, whereas overexpression of KLF4 promoted the phosphorylation of the PI3K/AKT signaling pathway (Fig. 5G and H). Collectively, these findings suggest that the knockdown of KLF4 inhibited the proliferation, migration, and invasion of CCA cells.

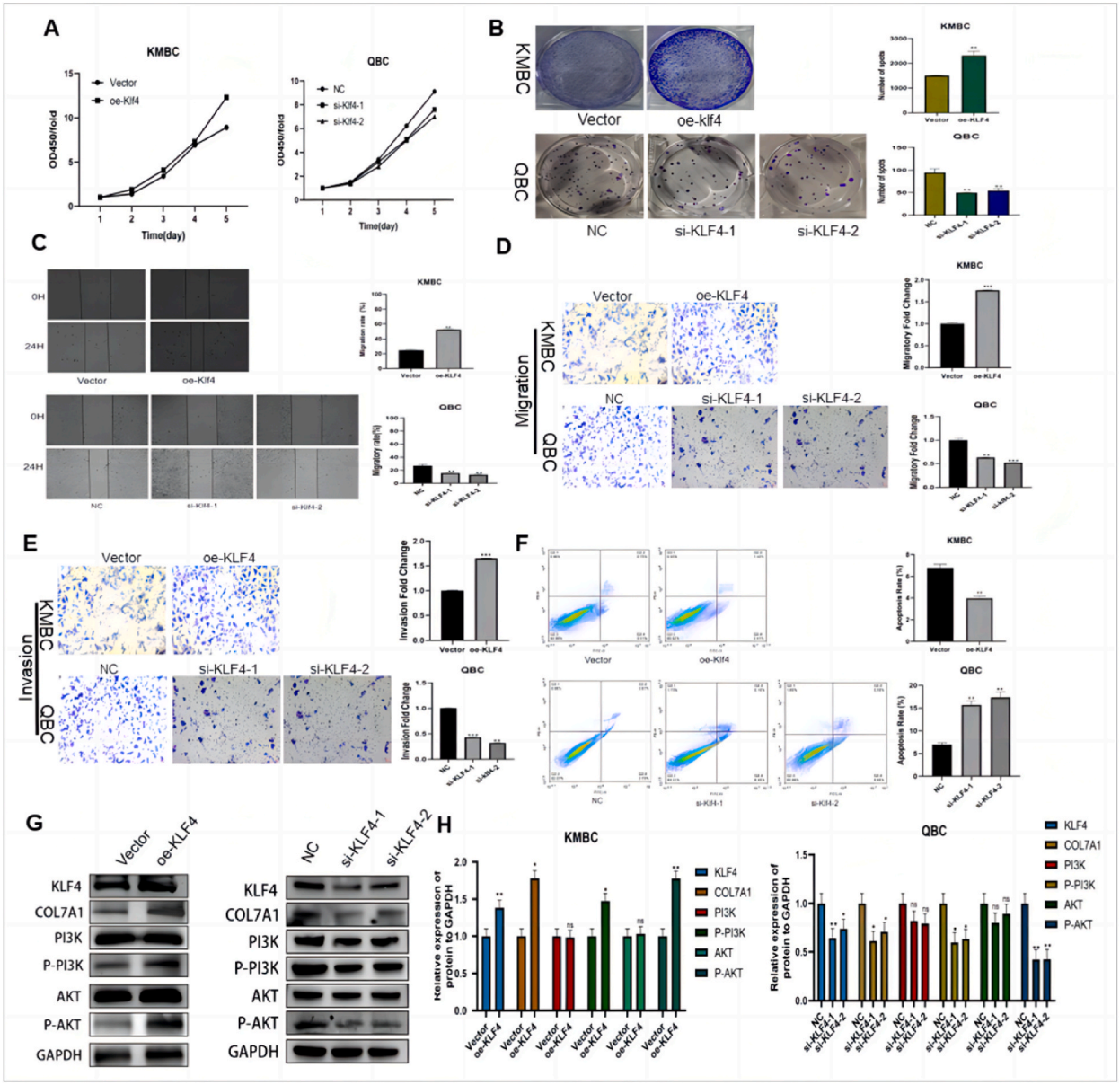

Fig. 5. KLF4 knockdown inhibits proliferation, migration, and invasion of CCA cells. (A–B) CCK8 and clone formation assay experiments were performed to detect the proliferation ability of KMBC and QBC939 cells. (C) Scratch assay to detect the migration ability of KMBC and QBC939 cells. (D–E) Transwell assay to detect the migration and invasion ability of KMBC and QBC939. (F) Flow cytometry assay to detect apoptosis of KMBC and QBC939 cells. (G–H) Western blotting analysis to detect PI3K/AKT protein levels after modulating KLF4 expression. Each set of experiments was performed in triplicate (The uncropped images are referenced in original blot: Fig. 5G). *p < 0.05, **p < 0.01, ***p < 0.001. CCA, cholangiocarcinoma.

3.6. *KLF4 inhibits the proliferation, migration, and invasion of CCA cells via the COL7A1/PI3K/AKT axis*

We constructed a model of COL7A1 knockdown model in KMBC cells overexpressing KLF4. We found that KLF4 overexpression could reverse the COL7A1 knockdown-mediated suppression of proliferation, migration, and invasion abilities of CCA cells (Fig. 6A–D). Overexpression of KLF4 with simultaneous COL7A1 knockdown induced insignificant changes in the expression of critical proteins of PI3K/AKT signaling, as detected by PI3K and AKT levels, and an increase in the protein expression of p-PI3K and p-AKT, which was reversed compared with cells with COL7A1 knockdown only (Fig. 6E). These findings suggested that KLF4 regulates the PI3K/AKT pathway through COL7A1 to inhibit the proliferation, migration, and invasion of CCA cells.

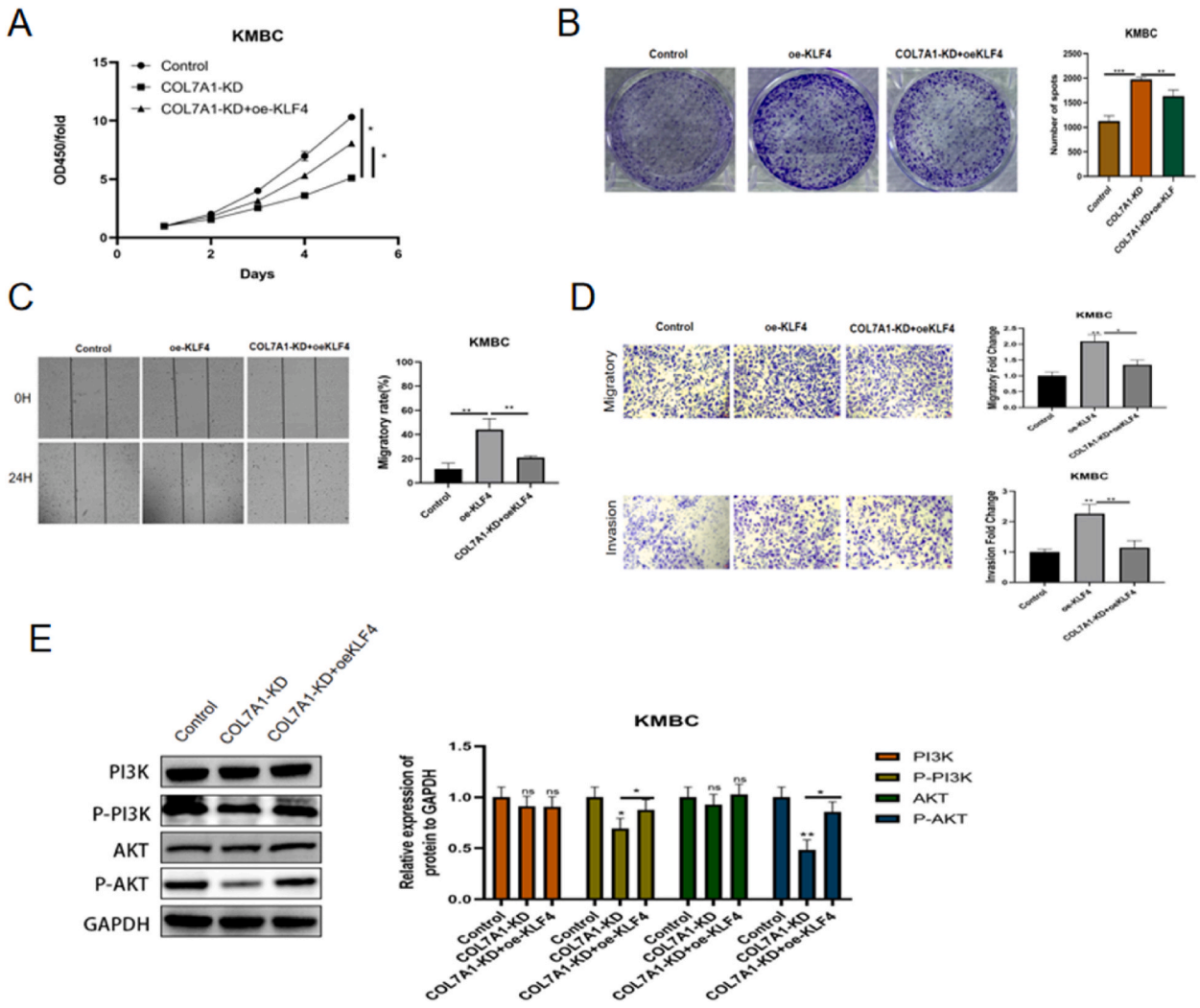

Fig. 6. KLF4 inhibits the COL7A1/PI3K/AKT axis and suppresses proliferation, migration, and invasion of CCA cells. (A–B) CCK8 and clone formation assays were performed to detect the proliferative capacity of KMBC and QBC939 cells. (C) Scratch assay to detect the migration capacity of KMBC and QBC939 cells. (D) Transwell assay to detect the migration and invasion ability of KMBC and QBC939. (E) Western blotting analysis to detect PI3K/AKT protein levels after modulating KLF4 expression. Each set of experiments was performed in triplicate (The uncropped images are referenced in original blot: Fig. 6E).*p < 0.05, **p < 0.01, ***p < 0.001. CCA, cholangiocarcinoma.

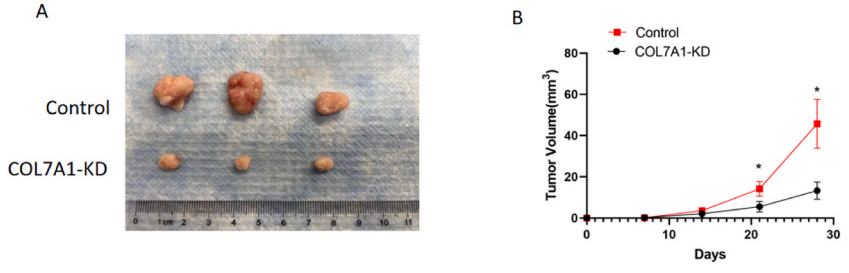

Fig. 7. Knockdown of COL7A1 *in vivo* inhibits tumor formation (A) Representative tumor image of a nude mouse tumorigenic model. (B) Tumor volume changes over time. *p < 0.05, **p < 0.01, ***p < 0.001.

3.7. Knockdown of COL7A1 in vivo inhibits tumor proliferation

To verify whether COL7A1 could inhibit CCA tumor growth *in vivo*, we injected the constructed COL7A1 knockdown stable transducer cells into nude mice and then observed and recorded tumor growth. The COL7A1 knockdown group exhibited slower tumor growth and a smaller tumor size than the control group (Fig. 7A and B). Therefore, COL7A1 knockdown in nude mice inhibits tumor growth.

4. Discussion

CCA is a specific biliary tract tumor with a very high five-year lethality rate owing to its early asymptomatic presentation, high aggressiveness, and treatment-resistant features[18,19]·It is crucial to identify CCA targets that can affect cell proliferation and invasion. COL7A1 is known to play multiple roles in cancer. For example, in squamous cell carcinoma of the skin, COL7A1 reportedly regulates the expression of signaling molecules such as PI3K and MAPK, which in turn promotes cell migration and invasive ability [16]. In our study of CCA, we found that COL7A1 was relatively highly expressed in CCA and regulated cell proliferation and migration. Furthermore, COL7A1 expression was found to substantially correlate with the survival prognosis of patients with gastric, laryngeal, and pancreatic cancers [20–22]. However, in CCA, high COL7A1 expression did not significantly correlate with patient survival, probably because of the relatively small number of patients with CCA in TCGA database. To examine the COL7A1-mediated regulation of the biological functions in CCA, we knocked down COL7A1 in CCA cells, which markedly suppressed the proliferation, invasion, and migration of CCA cells. These findings suggest that COL7A1 plays a pro-carcinogenic role in CCA development. COL7A1 can promote tumor growth by binding to the structural domain of laminin B3 and can activate PI3K phosphorylation to increase tumor invasion capacity [16,23]. Herein, we found that COL7A1 was involved in cellular tissue growth and wound healing, based on GO and KEGG analyses, and regulated the PI3K/AKT signaling pathway. The knockdown of COL7A1 decreased the protein levels of p-PI3K and p-AKT in cells, as evidenced in cellular experiments. Therefore, we hypothesized that COL7A1 regulates the phosphorylation of the PI3K/AKT signaling pathway.

Using the NCBI and UCSC databases, we predicted that KLF4 was the transcription factor of COL7A1. KLF4, as a common transcription factor, has a C-terminal end consisting of the end-binding protein and Sin3A, an evolutionarily conserved transcriptional corepressor that represses the transcriptional function of genes [24] Sin3A regulates both transcriptional repression and activation [25] which suggests that KLF4 exerts a dual regulatory function. We found that KLF4 binds to COL7A1 in CCA and that COL7A1 expression was positively correlated with the knockdown or overexpression of KLF4. This correlation was further confirmed using a dual-luciferase assay. KLF4 binds to COL7A1 in CCA and regulates COL7A1 expression, as well as regulates the PI3K/AKT signaling pathway in various cancers, affecting tumor cell proliferation and migration [26,27]. Following the knockdown and overexpression of KLF4 in CCA, protein levels of p-PI3K and p-AKT were altered and positively correlated. Therefore, we postulate that KLF4 can regulate the expression of COL7A1 and thus inhibit the phosphorylation and activation of the PI3K/AKT signaling pathway. Cell phenotyping experiments showed that KLF4 knockdown inhibited the proliferation and migration of CCA cells, and overexpression promoted the proliferation and migration of CCA cells. These results are consistent with the role of KLF4 in hepatocellular carcinoma and breast cancer [8,28]. Knocking down the expression of COL7A1 reversed the effects of KLF4 on the proliferation and migration of CCA cells, as well as the effect of KLF4 on the protein content of p-PI3K and p-AKT. Therefore, KLF4 may regulate the phosphorylation of the PI3K/AKT signaling pathway in CCA by regulating the expression of COL7A1, which, in turn, affects the proliferation and migration of CCA cells.

5. Conclusions

In conclusion, we demonstrated that KLF4, as a transcription factor, can regulate the phosphorylation of the PI3K/AKT signaling pathway by regulating the expression of COL7A1, which, in turn, affects the proliferation and migration of CCA cells. Our findings enrich our understanding of the molecular mechanisms underlying the role of COL7A1 in the pathogenesis of CCA.

Ethical approval

Ethical approval was obtained from the Ethics Committee of Bengbu Medical College (Nos. 2021230) and (No. 2021298). The study was conducted in accordance with the principles of the Declaration of Helsinki. Experiments were conducted in accordance with the ARRIVE guidelines for the care and use of animals.

Funding

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Data availability statement

No data associated with this study have been deposited in a publicly available repository. Data are available from the corresponding author upon request. The raw data was uploaded as additional files.

CRediT authorship contribution statement

Yang Ma: Writing – original draft, Investigation, Formal analysis. **Yanfang Zhang:** Investigation. **Fangfang Chen:** Investigation. **Sihua Liu:** Investigation. **Dongdong Wang:** Investigation, Formal analysis. **Zheng Lu:** Writing – review & editing, Conceptualization. **Dengyong Zhang:** Writing – review & editing, Conceptualization. **Rui Liang:** Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37361.

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