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# GATA1 gene silencing inhibits invasion, proliferation and migration of cholangiocarcinoma stem cells via disrupting the PI3K/AKT pathway

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**Background/aims:** Intrahepatic cholangiocarcinoma (CCA) is the second most prevalent type primary liver malignancy, accompanied by an increasing global incidence and mortality rate. Research has documented the contribution of the GATA binding protein-1 (GATA1) in the progression of liver cancer. Here, we aim to investigate the role of GATA1 in CCA stem cells via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway.

**Methods:** Initially, microarray-based gene expression profiling was employed to identify the differentially expressed genes associated with CCA. Subsequently, an investigation was conducted to explore the potential biological significance behind the silencing of GATA1 and the regulatory mechanism between GATA1 and PI3K/AKT pathway. CCA cell lines QBC-939 and RBE were selected and treated with siRNA against GATA1 or/and a PI3K/AKT pathway inhibitor LY294002. In vivo experiment was also conducted to confirm in vitro findings.

**Results:** GATA1 exhibited higher expression in CCA samples and was predicted to affect the progression of CCA through blockade of the PI3K/AKT pathway. siRNA-mediated downregulation of GATA1 and LY294002 treatment resulted in reduced proliferation, migration and invasion abilities of CCA stem cells, together with impeded tumor growth, and led to increased cell apoptosis and primary cilium expression. Additionally, the siRNA-mediated GATA1 downregulation had an inhibitory effect on the PI3K/AKT pathway. LY294002 was manifested to enhance the inhibitory effects of GATA1 inhibition on CCA progression. These in vitro findings were reproduced in vivo on siRNA against GATA1 or LY294002 injected nude mice.

**Conclusion:** Altogether, the present study highlighted that downregulation of GATA1 via blockade of the PI3K/AKT pathway could inhibit the CCA stem cell proliferation, migration and invasion, and tumor growth, and promote cell apoptosis, primary cilium expression.

**Keywords:** GATA1, gene silencing, cholangiocarcinoma stem cells, PI3K/AKT pathway, proliferation, migration, invasion

## Introduction

Cholangiocarcinoma (CCA) is a rare malignancy manifesting primarily in the epithelial cells in perihilar, intrahepatic and distal biliary tree.[1] The survival rate of patients with CCA is very low, and due to the typical late and limited treatment regimens, the five-year survival rate of patients suffering from CCA is less than 10%.[2] Patients with CCA exhibit no symptoms in early stages, but when they are diagnosed, there is high possibility that their disease has metastasized.[3] Existing literature has demonstrated the association of cancer stem cells (CSCs) with the

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occurrence, recurrence and metastasis of tumor and exhibit characteristics of both stem-like cells and higher chemotherapeutic resistance.[4] CSCs possess similar features related to normal stem cells and have properties to differentiate into all kinds of cell phenotypes of a specific cancer sample.[5] A recent study demonstrated the self-renewal and differentiation properties of CSCs in solid tumor and flagged CSCs to be principally responsible for the maintenance of tumor growth.[6] At present, surgical resection is the only medical intervention to treat intrahepatic CCA, which still presents with a poor prognosis.[7] Therefore, this raises concerns for the development of new therapeutic intervention for CCA.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is an important pathway, participating in the regulation of a variety of physiological processes in mammalian cells such as cell proliferation and tumor development; it induces tumor development as well as stimulates tumor metastasis.[8] The activated PI3K/AKT is essential for neuron apoptosis and autophagy while the inhibited PI3K/AKT pathway promotes hippocampal neurons apoptosis and decreases regular autophagy.[9] An existing study demonstrated the functionality of an abnormal expression of GATA binding protein-1 (GATA1) in the protection of megakaryocytes against activated PI3K/AKT stimulated apoptosis.[10] Previously, GATA binding protein-1 (GATA1) was originally identified as a transcription factor that binds to the b-globin regulatory region and it was later revealed as a member of the GATA transcription factor family, whose members bind the consensus "WGATAR" binding motif. GATA1 functions as a critical transcription factor to combine the common sequence GATA found in the modulatory areas of all erythroid specific genes.[11] GATA1 mediates the genes associated with erythroid and megakaryocytic cell progression, and its dysregulation is closely related with hematopoietic disorders.[12] GATA1 also functions as a key transcription factor that comprehensively regulates the development of erythroid and megakaryocytic cell and its deficiency could cause megakaryoblastic leukemia.[13,14] Previously, the functionality of GATA1 has been demonstrated in many multimeric complexes containing other transcription factors with emphasis on the activation or inhibition of the expression of target gene.[15] A former study concluded that upregulation of GATA1 contributed to the proliferation and apoptosis of cells.[16] Existing literature demonstrated that GATA1 could promote epithelial–mesenchymal transition (EMT) in breast cancer via activation of the PAK5 pathway.[17,18] In addition, GATA1 can promote the growth of tumor cells and may function as a potential indicator for breast cancer.[19] However, the role of GATA1 in the development of CCA is still unknown. In the present study, we performed a knockdown of the expression of GATA1 using the GATA1 siRNA and then examined whether downregulation of GATA1 could mediate the proliferation and apoptosis of CCA stem cells by means of the PI3K/AKT pathway.

# Materials and methods

## Ethics statement

Animal experiments were conducted under the approval of the Animal Ethics Committee of the Second Hospital of Jilin University. All animal experiments were performed in accordance with *the Guide for the Care and Use of Laboratory animals* published by the US National Institutes of Health. Appropriate measures were taken to minimize the use of animals as well as their suffering.

## Microarray-based gene expression profiling

"Cholangiocarcinoma" was searched as a keyword in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds/?term=) of the National Center for Biotechnology Information (NCBI) from which the GSE26566 dataset was finally selected. The dataset comprised 169 samples, which was a combination of 59 surrounding liver samples, 104 CCA samples and 6 normal intrahepatic bile duct samples. The sequencing platform was GPL6104. Differential expression analysis was performed on the CCA samples and normal intrahepatic bile duct samples in an attempt to analyze the expression of GATA1. The differential expression analysis for GATA1 was conducted in compliance with the Limma package R software. The threshold values were set as $p<0.05$ and | logFC | >2. After analysis, GATA1 was selected and its expression in samples was used to construct a box plot.

DisGeNET (http://www.disgenet.org/web/DisGeNET/menu) could be used to search for CCA-related genes.[20,21] "CCA" was searched as a keyword on this platform from which the top 10 genes with of highest relevance were selected for later analysis. STRING (https://string-db.org/) is a database that comprises reported and predicted protein interactions, which can be employed for analytical purposes of the interaction between proteins.

## Separation and identification of CCA stem cells

The separation of CCA stem cells was conducted as follows. CCA cell lines QBC939 (JN-A2042) and RBE (JN-A2657) were purchased from Shanghai Jining Shiye Co., Ltd. (Shanghai, China). Next, the above two cell lines were incubated with a combination of 10% fetal bovine serum (FBS, Sijiqing Bioengineering Materials Co., Ltd., Hangzhou, Zhejiang, China), 89.5% Dulbecco's modified eagle medium (DMEM, Hyclone Laboratories, Logan, UT, USA) and 0.5% penicillin and streptomycin solution at 37°C in an incubator with 5% $CO_2$. Then, cells in the logarithmic growth phase were trypsinized repeatedly and dissociated into single cell suspension, which was further inoculated into a six-well ultralow attachment plate to attain a cell density of $5\times10^3$ cells/mL (H23302, Yuanye Biotechnology Co., Ltd., Shanghai, China). Subsequently, the cells were incubated with a combination of serum-free DMEM/F12 (Hyclone Laboratories, Logan, UT, USA), epidermal growth factor (EGF) (20 ng/mL, Sigma-Aldrich Chemical Company, St Louis, MO, USA), basic fibroblast growth factor (bFGF) (20 ng/mL, Sigma-Aldrich Chemical Company, St Louis, MO, USA) and 4% stem cell culture additive B27 (Sigma-Aldrich Chemical Company, St Louis, MO, USA). After 10 days, the cell clone spheres were collected and cultured [illegible] new s[illegible]-well ultralow attachment plate for subculture. Then, the cell spheres were collected and dissociated into [illegible] cell suspension. Subsequently, the cells were incubated with 10 μL phycoerythrin (P[illegible])-anti-CD1[illegible] antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and 5 μL fluorescein isothiocyanate (FITC)-anti-epithelial-specific antigen ([illegible]A, BioLegend, San Diego, CA, USA) on ice for 20 mins and were then further separated by means of flow cytometry (FCM) procedure in order to select CD133+Ep $CAM^{high}$ stem cells.

Next, the selected CD133+Ep $CAM^{high}$ stem cells were cultured in [illegible] 96-well ultralow attachment plate (100 cells/well), and cultured in serum-free culture medium. Complete medium replacement was conducted every 2 days. Upon changing the medium, 60 μL of the upper culture medium from each well was precisely absorbed, and then 60 μL of fresh culture medium was supplemented to substitute for old medium. The formation of cell spheres in each well was observed frequently under an inverted microscope, photographed and recorded. The cell cluster containing more than 50 cells was acknowledged as 1 cell sphere.

The identification of selected CD133+Ep $CAM^{high}$ stem cells was conducted on the basis of a variety of parameters such as monoclonal formation rate, proliferative ability, drug resistance, protein expression of stem cell-related nuclear transcription factors OCT-4, Bmi-1, Vimentin and E-cadherin, and tumor-forming ability after subcutaneous transplantation of BALB/c mice.

## Silencing and validation of GATA1

Two small interfering RNA (siRNA) sequences for the GATA1 protein were designed and synthesized by Invitrogen Inc. (Carlsbad, CA, USA). Sequence 1: the sense sequence was UUCAUCUUGUGAUA[illegible]AGCCA and the antisense sequence was GCUU[illegible]AUCA[illegible]AGAU[illegible]AAUG (847). For the sequence 2, the sense sequence was UAGUUC AUAGCUACAUA[illegible]CUA and the antisense sequence was GCUAUGU[illegible]CUAU[illegible]ACUA[illegible]G (1475). After detachment, the cells were seeded [illegible] 24-well plate at a density of $5\times10^4$ cells/well, which was followed by the addition of 500 μL [illegible] complete culture medium. Under strict accordance with the manufacturer instructions, 12-hr cell transfection was conducted. The transfected cells were divided into 3 groups: the N[illegible] group (transfected with GATA1 NC sequence), the siRNA1 group (transfected with small interference sequence 1), and the siRNA2 group (transfected with small interference sequence 2). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis procedures were performed in an attempt to detect the mRNA and protein expression of GATA1 in the aforementioned 3 groups to verify the gene silencing.

## Cell treatment

The CCA stem cells were cultured in the RPMI 1640 medium (Hyclone Laboratories, Logan, UT, USA) containing 10% FBS and penicillin and streptomycin in an incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 5% $CO_2$ and saturated humidity at 37°C for 24 hrs in a sterile environment. The cells were divided into 6 groups, including the blank group (transfected with no sequence), the NC group (transfected with GATA1 NC sequence), the GATA1 group (transfected with GATA1 overexpressed vector, PLOC-GATA1 was purchased from Invitrogen Inc., Carlsbad, CA, USA), the GATA1 RNAi group (transfected with GATA1 RNAi), the LY294002 group (transfected with PI3K/AKT pathway inhibitor LY294002, 100 ng/mL, Sigma-Aldrich Chemical Company, St Louis, MO, USA) and the GATA1 RNAi + LY294002 group (co-transfected with GATA1 RNAi and PI3K/AKT pathway inhibitor LY294002 of 100 ng/mL). CCA stem cells in the logarithmic growth phase were seeded into a 6-well plate. Upon attaining a cell density of 30–50%, the cells were transfected under strict accordance with the instructions of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After performing cell culture at 37°C with 5% $CO_2$ for 6–8 hrs, the medium was replaced by complete medium. After culturing for 24–48 hrs, the planned follow-up experiments were conducted.

## RT-qPCR

The total RNA was extracted in strict accordance with the provided instructions of the RNA Extraction Kit (Invitrogen Inc., Carlsbad, CA, USA). The primers of GATA1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and synthesized by TaKaRa Biotechnology Co. Ltd. (Dalian, Liaoning, China) (Table 1). The RNA was reversely transcribed into cDNA in accordance with the provided instructions of the PrimeScript RT reagent kit. RT-qPCR was performed using the reaction solution according to the provided instructions of SYBR® Premix Ex Taq™ II kit. GAPDH was used as internal reference for this experiment. The relative expression of GATA1 was calculated based on the $2^{-\triangle Ct}$ method. The formula was as follows: $\triangle Ct = Ct_{(target\ genes)} - Ct_{(internal\ reference)}$, and the mRNA relative transcriptional levels of target genes = $2^{-\triangle\triangle Ct}$.[22]

## Western blot analysis

The total protein was extracted and the concentration was measured by performing bicinchoninic acid (BCA) assay. Next, 50 µg protein was separated by means of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. Membrane blockade was performed using 5% skim milk at room temperature for 1 hr, followed by overnight incubation with the following primary antibodies: rabbit polyclonal antibody to GATA1 (1:1000, ab28839, Abcam Inc., Cambridge, MA, USA), *p*-mTOR (1:1000, ser2448, #5536, Cell Signaling, Danvers, MA, USA), mTOR (1:1000, # 2983, Cell Signaling, Danvers, MA, USA), *P*-PI3K (1:1000, abs130868, Absin Bioscience Inc., Shanghai, China), PI3K (1:1000, abs119725, Absin Bioscience, Inc. Shanghai, China), *p*-AKT (1:1000, ab38449, Abcam Inc., Cambridge, MA, USA) and GAPDH (1:10,000, ab181602, Abcam Inc., Cambridge, MA, USA). After 3 rinses with phosphate buffer saline (PBS) solution at room temperature (5 mins/time), the membrane was incubated with the horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody immunoglobulin G (IgG, 1:[illegible],000, ab205[illegible], Abcam Inc., Cambridge, MA, USA) at 37°C for 1 hr. Next, the immunocomplexes on the membrane were visualized by the addition of HRP-labeled enhanced-chemiluminescence (ECL) reagent, which was followed by quantification of the band intensities using the Image J software. The experiment was repeated three times independently.

**Table 1** Primer sequences for RT-qPCR

| Gene | Sequence (5′- 3′) |
|---|---|
| *GATA1* | Forward primer: CAAGAAGCGCCTGATTGTCAG<br>Reverse primer: AGTGTCGTGGTGGTCGTCTG |
| GAPDH*GAPDH* | Forward primer: ATGCTGGCGCTGAGTACGTC<br>Reverse primer: CAGGGGTGCTAAGCAGTTGGT |

**Abbreviations:** RT-qPCR, reverse transcription quantitative polymerase chain reaction; GATA1, GATA binding protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## Cell counting kit-8 (CCK-8) assay

After 24 hrs of transfection, the cells were trypsinized into a single cell suspension. Next, the cells were seeded in a 96-well plate at a density of $3\times10^3$–$6\times10^3$ cells/well (200 µL/well). Six duplicate wells were set up. Then, cells were incubated in an incubator. The culture plates were taken out after cell culture for specific time points (24 hrs, 48 hrs and 72 hrs). CCK-8 (10 µL, Sigma-Aldrich Chemical Company, St Louis, MO, USA) was added to each well and repeatedly cultured for 2 hrs, followed by detection of the optical density (OD) value at 450 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (NYW-96M, Beijing Nuoyawei Instrument Technology Co., Ltd., Beijing, China). Each experiment was repeated three times independently. Cell viability curve was drawn with time as the abscissa and OD value as the ordinate.

## Scratch test

CCA stem cells transfected for 24 hrs were seeded in a 6-well plate. After reaching complete confluence, cells were gently scrapped off in the center axis of the plate with a 10 µL pipette tip. After a rinse and removal using PBS solution, the floating cells were continuously cultured for 24 hrs, observed and photographed under a microscope. The migration ability of cells was expressed as the percentage of scar healing. The percentage of cell healing = (scratch width before experiment - scratch width after culture for 24 hrs)/scratch width before experiment ×100%.

## Transwell assay

After 48 hrs of transfection, the stem cells were resuspended in DMEM containing 1% FBS and the cell density was adjusted to $1\times10^5$–$10\times10^5$ cells/mL. Next, 200 μL of cell suspension was added to the Transwell chamber (24-well plate), while DMEM containing 10% FBS was added to the lower chamber. The cells were cultured in a 37°C incubator and the chamber was removed after 24 hrs, fixed using ethanol for 30 mins and then finally stained using 0.1% crystal violet for 20 mins. The cells were randomly selected from 5 fields under a 400× light microscope for observation and counting.

## Flow cytometry

After 96 hrs of transfection, the cells were detached by ethylenediaminetetraacetic acid (EDTA)-free trypsin and collected. After 2 rinses in precooled PBS solution at 4°C, the supernatant was discarded. The cells were resuspended in binding buffer, with adjustment of the cell density to approximately $1\times10^6$ cells/mL. Cell suspension (100 μL) was transferred to a flow tube, which was followed by the addition of 5 μL of calcium ion-dependent phosphate-binding protein and 5 μL of propidium iodide (PI, 1 mg/mL), gently shaken and allowed to react in conditions devoid of light at room temperature for 15 mins. Finally, 400 μL of 1× binding buffer was added to the cells and detected using a flow cytometer within 1 hr.

## Immunofluorescence assay

CCA stem cells with proper density were seeded in a cover glass. After stimulation of different interventions for 24 hrs, the cells were fixed using 4% polyformaldehyde for 15 mins, blocked at room temperature using 0.5% bovine serum albumin (BSA) for 60 mins and finally incubated with the antibody to acetylated alpha-tubulin (in a ratio of 1:300, Abcam Inc., Cambridge, MA, USA) from mouse overnight. After 3 rinses with PBS solution, the cells were incubated at room temperature for 1 hr with the CY3-labeled goat anti-mouse fluorescence secondary antibody. The nuclei were stained with 1 μg/μL 4′,6-diamidino-2-phenylindole (DAPI) and rinsed 3 times with PBS solution, after which the experiment results were observed under a fluorescence microscope.

## Limiting dilution assay (LDA) in vivo

The cells were seeded in a low attachment culture plate for 7 days. Then, the CCA stem cell spheres from each group were collected, transferred to 10 mL glass centrifuge tubes, detached for 10 mins and centrifuged. Next, the cells were resuspended in normal saline and counted. Then, a different number of cells ($1\times10^3$, $5\times10^3$, $1\times10^4$, $5\times10^4$) were resuspended in 50 μL of normal saline, and then further mixed with 50 μL of Matrigel Matrix in the ratio of 1:1 and inoculated to NOD-SCID mice subcutaneously. Two weeks after inoculation, the tumor formation was observed and documented. The proportion of tumor stem cells was calculated by means of ELDA (http://bioinf.wehi.edu.au/software/elda/index.html).[23]

## Tumor formation in nude mice

The CCA stem cells (QBC-939) were collected to prepare a single cell suspension. An equal volume mixture of PBS and Matrigel prepared in the ratio of 1:1 was used to suspend the cells and dilute the cell concentration to $1\times10^6$ cells/200 μL. The 18 specific pathogen-free nude mice were divided into 6 groups (the blank, NC, GATA1, GATA1 RNAi, LY294002 and GATA1 RNAi + LY294002 groups), with 3 mice in each group. The nude mice in the LY294002 and GATA1 RNAi + LY294002 groups were administered with the dosage of 10 mg/kg once a week for 4 consecutive weeks. After anesthetizing the nude mice of each group with 3% pentobarbital sodium, 200 μL of CCA stem cell suspension was injected into the subcutaneous portion of the right hind leg of mice. The mice were raised under the same environment, observed once a week with in-depth documentation of the length and width of the tumor. The volume of the tumor was calculated according to the formula: the volume=length×width$^2$/2. On the 28th day, the mice were euthanized and the tumors were removed, with 6 tumors in each group.

## Spheroids formation assay

The cells were seeded into a 96-well low attachment plate at a density of $1\times10^4$ CCA stem cells/well and cultured in serum-free DMEM-F12 culture medium containing 20 ng/mL EGF and 20 ng/mL FGF-β. Half of the medium was changed every 2 days. After culturing for 10 days, the cells were observed, photographed and counted under a microscope.

## Statistical analysis

Statistical analyses were conducted using the SPSS 21.0 software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as means±standard deviation. The count data were expressed as a percentage or a ratio. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, while comparisons between multiple groups at different time points were conducted using repeated measures ANOVA. Comparisons between two groups were analyzed by independent sample *t*-test. A value of $p<0.05$ was indicative of a statistically significant difference.

## Results

### The expression of GATA1 and protein interaction networks in CCA

The GEO database was used to screen out the samples of CCA patients, from which the GSE26566 dataset was selected. Differential analysis performed on the dataset showed that the expression of GATA1 in CCA samples was significantly higher than the expression in normal samples (Figure 1A). Genes relative to CCA were retrieved through the online database, and the top 10 genes with the highest relevancy were selected for subsequent analysis (Table 2). Moreover, researches on CCA provide evidence supporting the functionality of the PI3K/AKT pathway in the progression of CCA. For instance, previous studies demonstrated that Osthole could induce apoptosis and inhibit the proliferation of CCA cells via the PI3K/AKT pathway.[24] Long-non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (lncRNA-MALAT1) can promote the proliferation and invasion of CCA cells by means of activation of the PI3K/AKT pathway.[25] Besides, existing literature revealed that activation of the PI3K/AKT pathway promotes the development of CCA.[26–] STRING was performed in an attempt to analyze the protein interaction of the CCA-related genes GATA1, PIK3R1 and other genes of the PI3K/AKT pathway. The results illustrated the existence of a direct interaction between GATA1 and PIK3R1 and also highlighted that it can directly influence

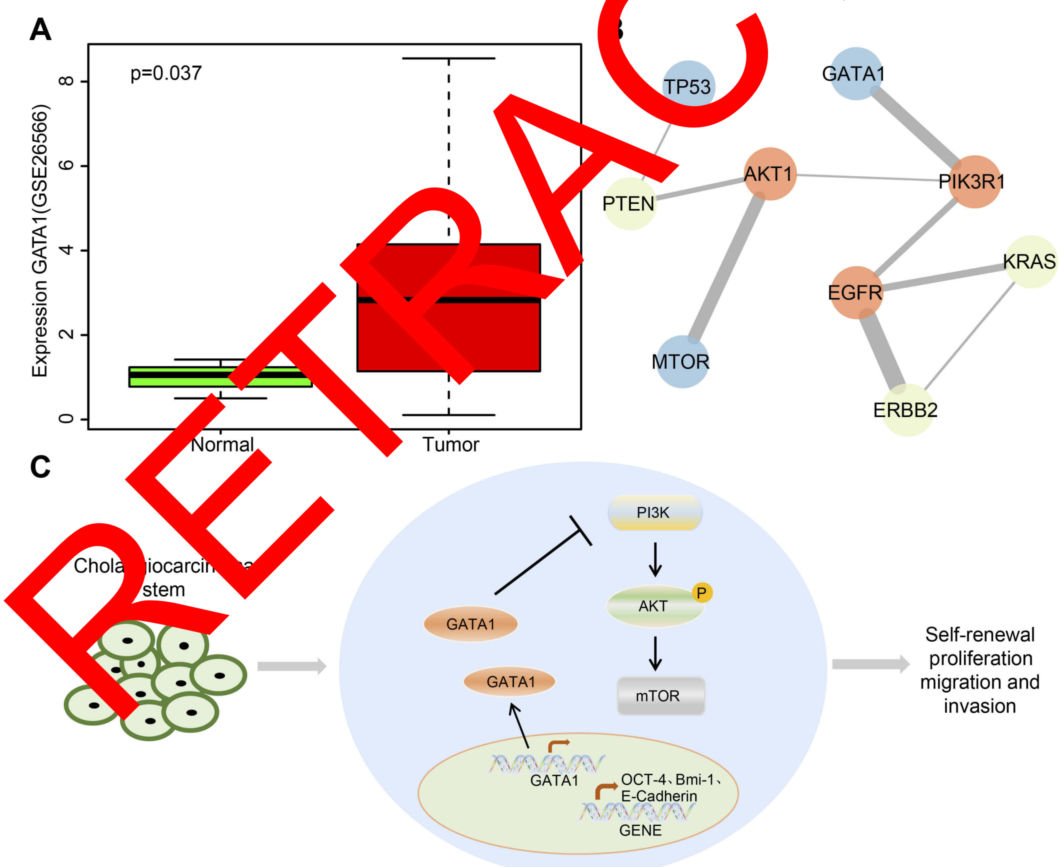



**Figure 1** GATA1 affects the onset of CCA via regulation of the PI3K/AKT pathway. (**A**) The green box diagram represents the sequencing expression of the gene in normal samples, the red box diagram indicates the sequencing expression of GATA1 in CCA samples and *p* is obtained by using the R language "Limma" package for differential expression calculation for GATA1; (**B**) the color of the round represents the core level of the protein, the deeper color reflects the core level and the thickness of the lines indicates the reliability of the interaction between the two proteins; thicker line reflects the reliability of the interaction; (**C**) mechanism map of GATA1 in CCA stem cells.

**Table 2** Retrieval of CCA-related genes (Top 10)

| Gene | Gene name | Score | PMIDs |
|---|---|---|---|
| TP53 | Tumor protein p53 | 0.291 | 24 |
| PTGS2 | Prostaglandin-endoperoxide synthase 2 | 0.215 | 19 |
| EGFR | Epidermal growth factor receptor | 0.213 | 21 |
| ERBB2 | Erb-b2 receptor tyrosine kinase 2 | 0.211 | 14 |
| IL6 | Interleukin 6 | 0.208 | 18 |
| KRAS | KRAS proto-oncogene, GTPase | 0.205 | 13 |
| SLC5A5 | Solute carrier family 5 member 5 | 0.203 | 1 |
| GNAS | GNAS complex locus | 0.203 | 2 |
| PTEN | Phosphatase and tensin homolog | 0.201 | 6 |
| IDH2 | Isocitrate dehydrogenase (NADP (+)) 2, mitochondria | 0.201 | 5 |

**Notes:** Score of the reliability of the gene-disease pair, based on the type and number of sources where is reported, and the number of PMIDs; PMIDs: Total number of PMIDs supporting the association.
**Abbreviation:** CCA, cholangiocarcinoma.

the expression of CCA-related genes (Figure 1B). Moreover, Stankiewicz et al reported that GATA1 stimulates the activation of AKT and thereby affects cells.[10] Altogether, we could conclude that GATA1 played a role in the PI3K/AKT pathway and thereby affected the biological features of CCA stem cells (Figure 1C).

## CAM$^{high}$ stem-like cells are successfully separated

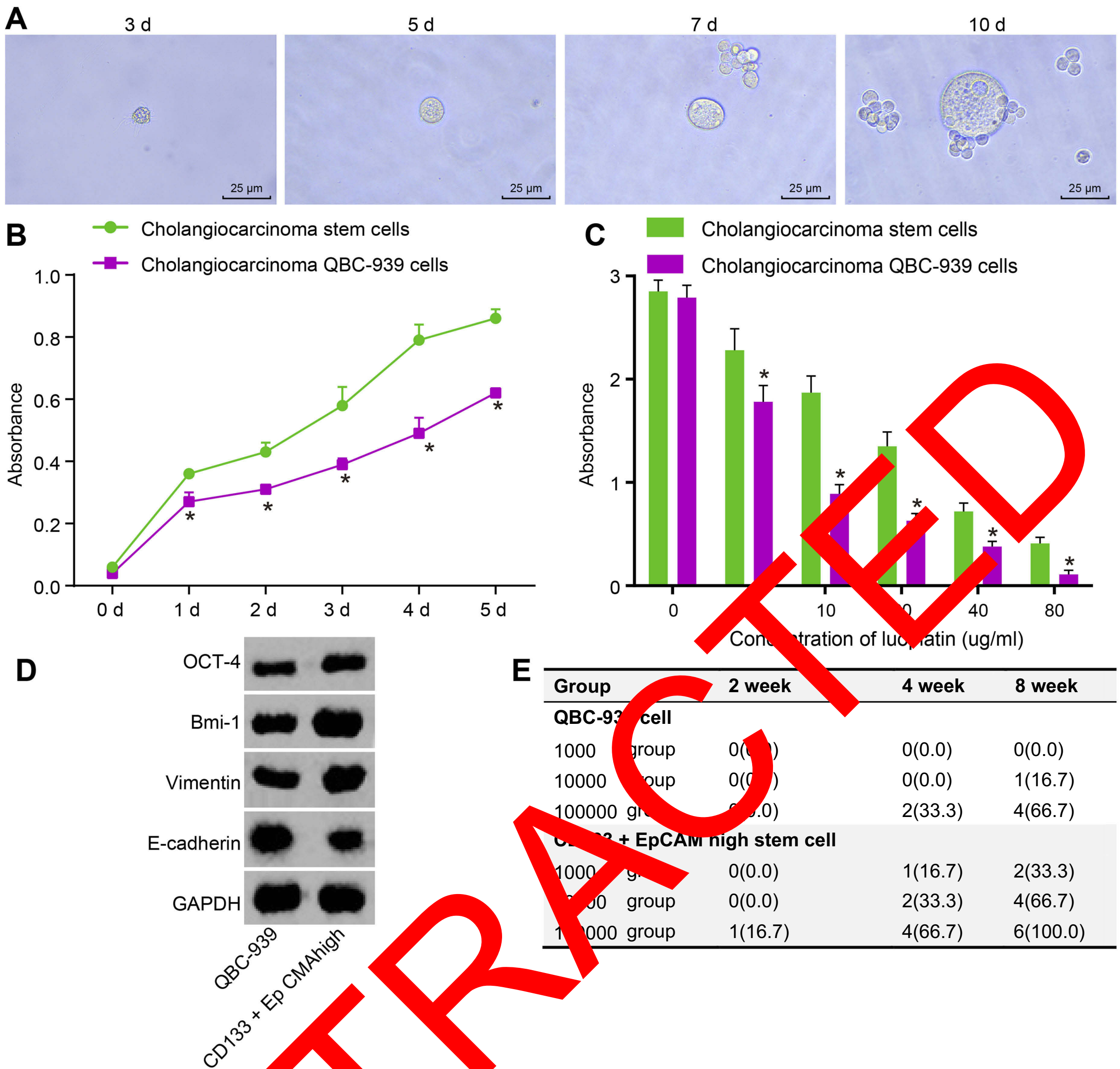
The separated QBC-939 cells were selected and cultured in serum-free medium. At the 10th day, spheres with tens of cells and tight junctions were observed under microscopic examination. In comparison with the QBC-939 cells, the CD133+Ep CAM$^{high}$ stem-like cells exhibited a significantly increased monoclonal formation rate ($p$<0.05) (Figure 2A). The OD values of cells in each group were measured. On comparing with QBC-939 cells, it was observed that the CD133+Ep CAM$^{high}$ stem-like cells had higher OD values, increased stem-like cell number and stronger proliferative ability ($p$<0.05) (Figure 2B). The OD values of cells in each group were compared. The results of OD value comparison depicted that upon administration of equal (less than 40 µg/mL) concentrations of lobaplatin (a drug for separating CAM$^{high}$ stem-like cells) the CD133+Ep CAM$^{high}$ stem-like cells had a significantly higher OD value and better drug resistance than the QBC-939 cells ($p$<0.05) (Figure 2C). The results of Western blot analysis were evident for a significantly increased expression of stem cell-related nuclear transcription factors in CD133+Ep CAM$^{high}$ stem-like cells: relative to the QBC-939 cells, OCT-4 expression was approximately 1.62 times higher; Bmi-1 was approximately 2.4 times higher; Vimentin was approximately 1.9 times higher; while the E cadherin content was lower, approximately 0.51 times higher (Figure 2D). The CCA cells of BALB/c female mice showed that QBC-939 cells could not exercise its tumorigenic properties when cell concentration reached $10^3$, while only 1 mouse was tumorigenic upon the culturing of the CD133+Ep CAM$^{high}$ stem-like cells for 4 weeks at the density of $10^3$, with highlighting that the tumor formation rate was 100% when the cell concentration reached $10^5$. The tumor formation rate of CD133+Ep CAM$^{high}$ stem-like cells was obviously higher in vivo than the CCA QBC-939 cells ($p$<0.05) (Figure 2E). The CAM$^{high}$ stem-like cells separated from REB cells exhibited similar results with those from the QBC-939 cells (Figure S1).

## siRNA1 and siRNA2 sequences could silence GATA1

The efficiency of GATA1 silencing in CCA stem cells from QBC-939 cells was examined by means of RT-qPCR and Western blot analysis procedures. As presented in Figure 3A, compared with the NC group, the siRNA1 and siRNA2 groups demonstrated significantly decreased mRNA expression of GATA1 ($p$<0.05). Results of Western blot analysis are shown in Figure 3B and C. In comparison with the NC group, the siRNA1 and siRNA2 groups exhibited a significantly reduced protein expression of GATA1 ($p$<0.05). The results demonstrated that both siRNA1 and siRNA2 sequences could effectively reduce the mRNA and protein expression of GATA1 and thereby silence GATA1. The results of RT-qPCR and Western blot analysis conducted on CCA stem cells from REB showed similar results (Figure S2).

## siRNA-mediated downregulation of GATA1 inhibits proliferation, invasion and migration and promotes apoptosis of CCA stem cells by blocking the PI3K/AKT pathway

In an attempt to explore the potential effects of GATA1 on the PI3K/AKT pathway in CCA stem cells QBC-939, Western blot analysis was conducted. As shown in Figure 4 A-B, no significant difference was observed in the expression of GATA1, as well as the extent of PI3K, AKT and mTOR phosphorylation between the blank group and the NC group ($p$>0.05). In comparison with the blank




| Group | 2 week | 4 week | 8 week |
|---|---|---|---|
| QBC-93[illegible] cell | | | |
| 1000 group | 0([illegible]) | 0(0.0) | 0(0.0) |
| 10000 group | 0([illegible]) | 0(0.0) | 1(16.7) |
| 100000 group | [illegible].0) | 2(33.3) | 4(66.7) |
| [illegible] + EpCAM high stem cell | | | |
| 1000 group | 0(0.0) | 1(16.7) | 2(33.3) |
| [illegible]00 group | 0(0.0) | 2(33.3) | 4(66.7) |
| 1[illegible]000 group | 1(16.7) | 4(66.7) | 6(100.0) |

**Figure 2** CAM$^{high}$ stem-like cells from QBC-939 cells have higher monoclonal formation rate, OD value and tumor formation rate. (**A**) Monoclonal formation images of CD133+Ep CAM$^{high}$ stem-like cells from QBC-939 cells; (**B**) OD values of CD133+Ep CAM$^{high}$ stem-like cells from QBC-939 cells; (**C**) OD values of CD133+Ep CAM$^{high}$ stem-like cells from QBC-939 cells when the concentration of Lobaplatin was the same; (**D**) protein expression of CD133+Ep CAM$^{high}$ stem-like cells from QBC-939 cell-related nuclear transcription factors; (**E**) tumor formation rate of CD133+Ep CAM$^{high}$ stem-like cells from QBC-939 cells. *$p<0.05$ *vs* QBC-939 cells. Measurement data were expressed as means±standard deviation. The count data were expressed as a percentage or a ratio. Comparison between multiple groups at different time points was conducted using repeated measures ANOVA. Comparisons between two groups were analyzed by independent sample *t*-test.



and NC groups, the GATA1 group showed obviously increased protein expression of GATA1, as well as the extent of PI3K, AKT and mTOR phosphorylation ($p<0.05$), while the si-GATA1 group demonstrated an opposite trend. No definitive difference was evident in terms of the GATA1 expression in the LY294002 group ($p>0.05$). The extent of PI3K, AKT and mTOR phosphorylation in the LY294002 group decreased remarkably ($p<0.05$). The si-GATA1 + LY294002 group illustrated remarkably decreased protein expression of GATA1, as well as the extent of PI3K, AKT and mTOR phosphorylation ($p<0.05$). In comparison with the si-GATA1 group, the LY294002 group displayed significantly increased protein expression of GATA1 ($p<0.05$), while no significant difference was detected in the extent of PI3K, AKT and mTOR phosphorylation ($p>0.05$). Relative to the si-GATA 1 group, the si-GATA1+LY294002 group illustrated no significantly different protein expression of GATA1 ($p>0.05$), whereas significantly reduced extent of PI3K, AKT and mTOR phosphorylation was observed ($p<0.05$). These results hypothesize positive inactivation of the PI3K/AKT pathway upon downregulation of GATA1.

Subsequently, six groups of QBC-939 cells were seeded in a 96-well plate (Figure 4C). The cell

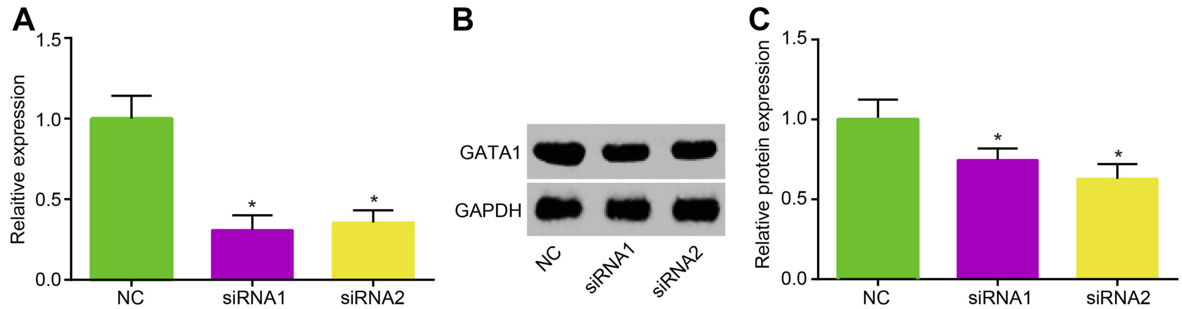



**Figure 3** siRNA1 and siRNA2 effectively silenced GATA1. (**A**) The mRNA expression of GATA1 in stem cells from QBC-939 cells in response to NC, siRNA1 and siRNA2 measured by RT-qPCR; (**B** and **C**) the protein bands and expression of GATA1 in stem cells from QBC-939 cells in response to NC, siRNA1 and siRNA2 measured by Western blot analysis. *$p<0.05$ vs the NC group. Measurement data were expressed as means±standard deviation. Comparisons between multiple groups were assessed by one-way ANOVA followed by Tukey post hoc test.



proliferation was observed by CCK-8 at specific time points (24 hrs, 48 hrs and 72 hrs), from which the relative proliferation rate of cells was calculated. In comparison with the cell proliferation rate at 0 hr, cell proliferation rates at 24-hr, 48-hr and 72-hr time points were significantly different (all $p<0.05$). On comparing with the blank and NC groups, it was observed that the GATA1 group had a significantly higher cell proliferation rate ($p<0.05$), while the si-GATA1 and LY294002 groups had lower cell proliferation rate ($p<0.05$), with the si-GATA1+LY294002 group presenting with the lowest cell proliferation rate ($p<0.05$). No significant difference was evident between the si-GATA1 group and the LY294002 group ($p>0.05$). In comparison with the si-GATA1+LY294002 group, the LY294002 group showed a higher cell proliferation rate ($p<0.05$). The results showed that silencing GATA1 along with inactivation of the PI3K/AKT pathway could inhibit CCA cell proliferation.

Afterward, the scratch test and Transwell assay were performed in order to elucidate the conceivable effects of GATA1 on the migration and invasion abilities of CCA stem cells QBC-939 (Figure 4D–G). No significant difference was observed in terms of the migration and invasion abilities of CCA stem cells between the blank group and the NC group ($p>0.05$). In comparison with the blank and NC groups, the si-GATA1 and LY294002 groups displayed significantly decreased migration and invasion abilities of cells (all $p<0.05$). The si-GATA1+LY294002 group presented with most significantly decreased migration and invasion abilities ($p<0.05$), while the migration and invasion abilities of the GATA1 group increased significantly ($p<0.05$). No statistical difference was evident between the si-GATA1 group and the LY294002 group ($p>0.05$). In comparison with the si-GATA1+LY294002 group, the LY294002 group showed increased migration and invasion abilities of cells ($p<0.05$). This data conjointly concludes that inhibiting GATA1 and the PI3K/AKT pathway can disrupt CCA cell migration and invasion.

Furthermore, Annexin V/PI double staining was performed so as to detect the effects of GATA1 on CCA cell apoptosis (Figure 4H and I). The apoptosis rates of the blank, NC, GATA1, si-GATA1, LY294002 and si-GATA1+LY294002 groups were (32.65±1.68)%, (35.2[illegible]8)%, (23.13±2.75)%, (44.54±1.16)%, (46.80[illegible]32)% and (54.89±3.82)%, respectively. No significant difference was illustrated in the apoptosis rate between the blank group and the NC group ($p>0.05$). In comparison with the blank and NC groups, the GATA1 group showed significantly decreased apoptosis rate ($p<0.05$), while the si-GATA1 and LY294002 groups demonstrated significantly increased apoptosis rate (all $p<0.05$), with the si-GATA1 + LY294002 group exhibiting the most significantly increased apoptosis rate ($p<0.05$). No significant difference was observed in the apoptosis rate between the si-GATA1 and LY294002 groups ($p>0.05$). In comparison with the si-GATA1+LY294002 group, the LY294002 group exhibited a significantly decreased apoptosis rate ($p<0.05$). These results propose that silencing GATA1 and inhibiting PI3K/AKT pathway can promote the apoptosis of CCA stem cells.

The results of the aforementioned experiments conducted on stem cells from CCA cell line REB demonstrated similar results as those obtained in the QBC-939 cells (Figure S3A–I). Conjointly, silencing of GATA1 induced inhibited proliferation, invasion and migration and enhanced the apoptosis of CCA stem cells by repressing the PI3K/AKT pathway.

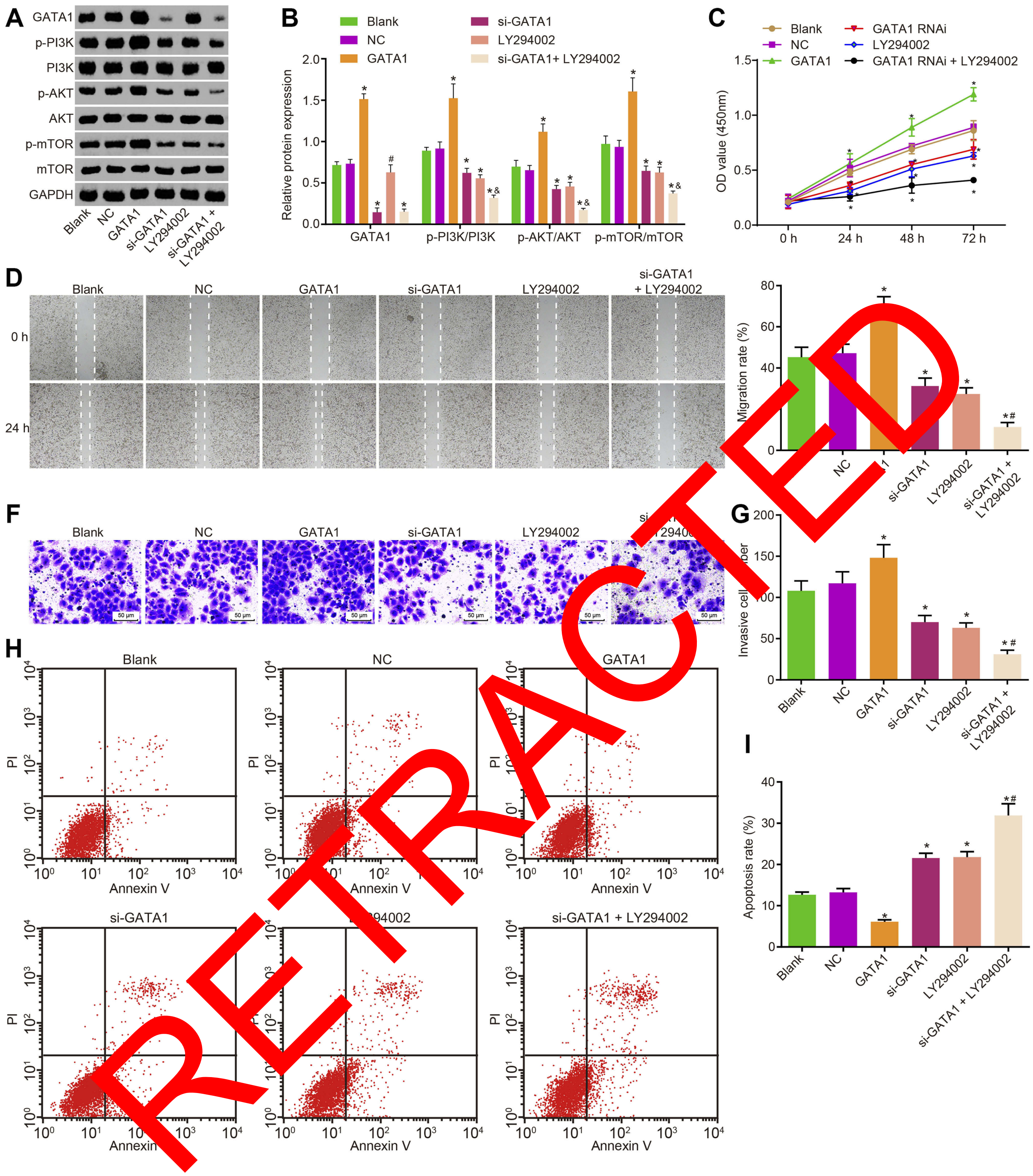



**Figure 4** siRNA-mediated downregulation of GATA1 suppresses invasion, proliferation and migration and promotes apoptosis of CCA stem cells through inactivation of the PI3K/AKT pathway. Stem cells from QBC-939 cells were treated with GATA1, si-GATA1, LY294002, si-GATA1+LY294002. (**A** and **B**) Protein bands and expression of GATA1 as well as the extent of PI3K, AKT and mTOR phosphorylation in stem cells from QBC-939 cells measured by Western blot analysis; (**C**) cell proliferation rate at 24 hrs, 48 hrs and 72 hrs in stem cells from QBC-939 cells assessed by CCK-8; (**D** and **E**) cell migration ability of stem cells from QBC-939 cells detected by scratch test (×40); (**F** and **G**), cell invasion ability of stem cells from QBC-939 cells evaluated by Transwell assay (×200); (**H** and **I**) cell apoptosis of stem cells from QBC-939 cells examined by Annexin V/PI double staining; *$p<0.05$ vs the blank and NC groups; #$p<0.05$ vs the si-GATA1 group; &$p<0.05$ vs the LY294002 group. Measurement data were expressed as means±standard deviation. Comparisons between multiple groups were assessed by one-way ANOVA followed by Tukey post hoc test.

## siRNA-mediated downregulation of GATA1 promotes primary cilium expression of CCA stem cells

The characteristics of primary cilium following GATA1 treatment have been presented in Figure 5. These results were depictive of no significant difference in the primary cilium expression between the blank and NC groups ($p>0.05$). In comparison with the NC and blank groups, the si-GATA1 and LY294002 groups had significantly increased primary cilium expression ($p<0.05$), with the si-GATA1+LY294002 group presenting with the most significantly increased expression ($p<0.05$). The expression of primary cilium in the GATA1 group was significantly decreased ($p<0.05$). No statistical difference was observed in terms of the primary cilium expression between the si-GATA1 and LY294002 groups ($p>0.05$). In comparison with the si-GATA1 +LY294002 group, the LY294002 group displayed a notably reduced primary cilium expression ($p<0.05$). The results showed that inhibition of GATA1 and a blockade of the PI3K/AKT pathway could promote the expression of primary cilium, maintain the integrity of cilium structure and inhibit the proliferation and invasion of CCA stem cells.

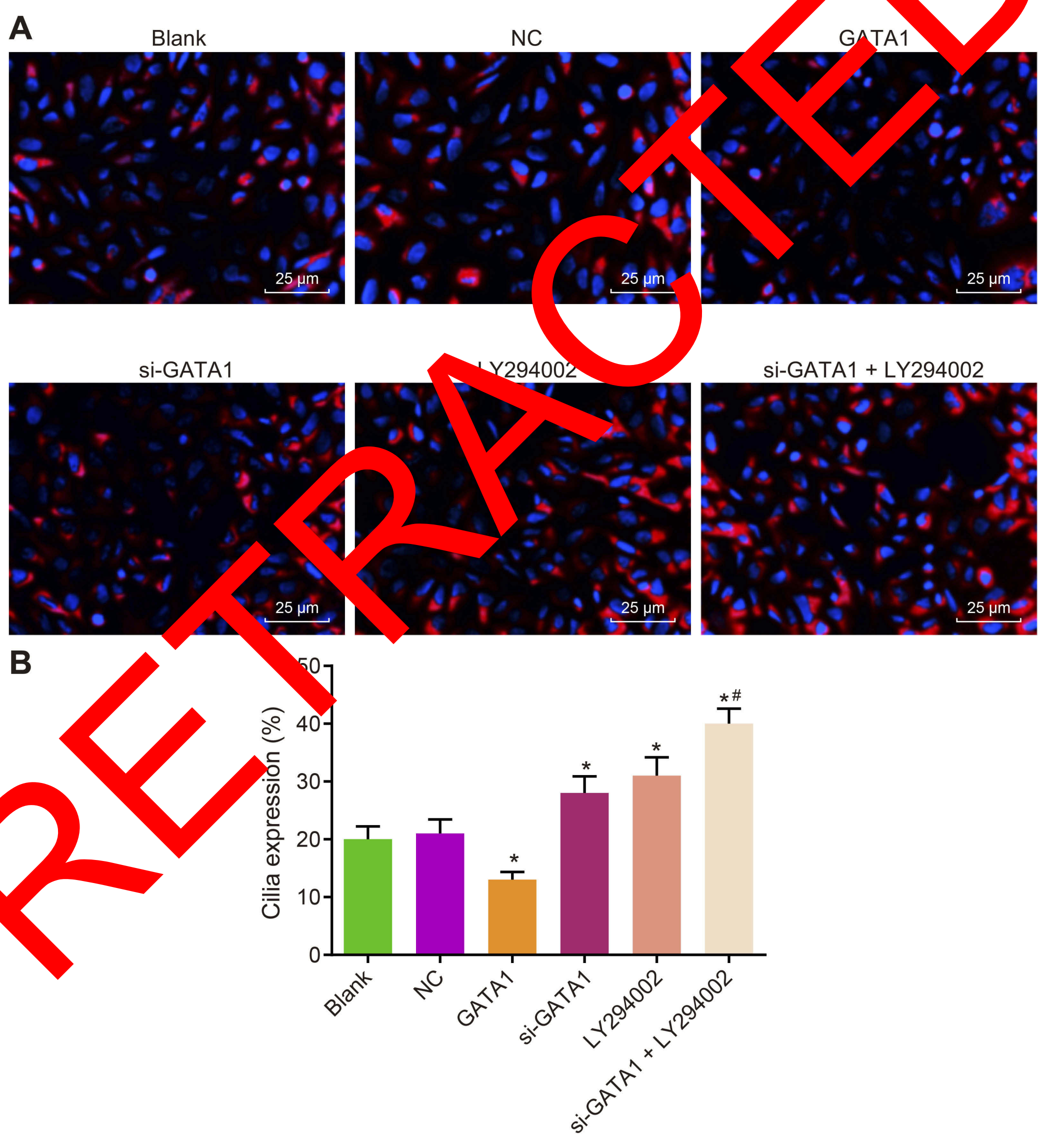



**Figure 5** siRNA-mediated downregulation of GATA1 drives the primary cilium expression. (**A**) The primary cilium on the surface of CCA stem cells stained with red fluorescence observed by fluorescence staining, the morphology of the primary cilium was observed under different intervention conditions and nucleus was stained with blue by DAPI solution (×400); (**B**) the expression of the primary cilium in response to the treatment of GATA1, si-GATA1, LY294002 and si-GATA1+LY294002; *$p$<0.05 vs the blank and NC groups; #$p$<0.05 vs the si-GATA1 and LY294002 groups. Measurement data were expressed as means±standard deviation. Comparisons between multiple groups were assessed by one-way ANOVA followed by Tukey post hoc test.

## siRNA-mediated downregulation of GATA1 inhibits tumor initiation and growth in vivo

The potential effects of GATA1 on tumor progression were evaluated by performing LDA in vivo, the results of which presented with a higher number of tumors and proportion of CCA stem cells in the GATA1 group than that in the blank and NC groups, while the same values in the si-GATA1, LY294002 and si-GATA1+LY294002 groups were lower (Table 3). No statistical difference was observed for the aforementioned parameters between the si-GATA1 and LY294002 groups ($p>0.05$). In comparison with the si-GATA1+LY294002 groups, the LY294002 group illustrated a higher number of tumors formed and proportion of CCA stem cells ($p<0.05$). These results indicated that silencing of GATA1 inhibited tumor initiation in vivo.

The tumor sizes of mice in the blank, NC, GATA1, si-GATA1, LY294002 and si-GATA1+LY294002 groups were measured every three days using vernier calipers, from which the growth curve of the transplanted tumor was plotted. As shown in Figure 6, compared with the NC and blank groups, the tumor growth rate in the si-GATA1, LY294002, si-GATA1+LY294002 groups decreased 2 weeks after transfection, while the rate in the GATA1 group was stimulated 2 weeks after transfection, with significant progression in the tumor rate over time ($p<0.05$). The mice were euthanized 4 weeks later, and the tumor tissues were extracted and the volumes of transplanted tumors were calculated. The tumor volumes of the si-GATA1 and LY294002 groups were significantly lower than the volumes observed in the NC and blank groups, with the si-GATA1+LY294002 group illustrating the most significant inhibition of tumor volume. No significant difference was observed between the si-GATA1 and LY294002 groups ($p>0.05$). In comparison with the si-GATA1+LY294002 group, the LY294002 group had a significantly increased volume of tumor ($p<0.05$). These results highlighted the inhibitory effect of silenced GATA1 on tumor growth via blockade of the PI3K/AKT pathway.

**Table 3** The proportion of CCA stem cells treated with GATA1, si-GATA1, LY294002 and si-GATA1+LY294002 in vivo

| Injected cells | Blank | NC | GATA1 | si-GATA1 | LY294002 | si-GATA1+LY294002 |
|---|---|---|---|---|---|---|
| 1,000,000 | 5/6 | 5/6 | 6/6 | [illegible] | 4/6 | 3/6 |
| 100,000 | 4/6 | 3/6 | [illegible] | 3/6 | 2/6 | 2/6 |
| 10,000 | 3/6 | 2/6 | 5/6 | 2/6 | 2/6 | 2/6 |
| 1,000 | 0/6 | 1/6 | 4/6 | 1/6 | 0/6 | 1/6 |
| Total | 12/24 | [illegible] | [illegible] | 10/24 | 8/24 | 8/24 |

**Abbreviations:** CCA, cholangiocarcinoma; GATA1, GATA binding protein-1; si, small interfering; NC, negative control.

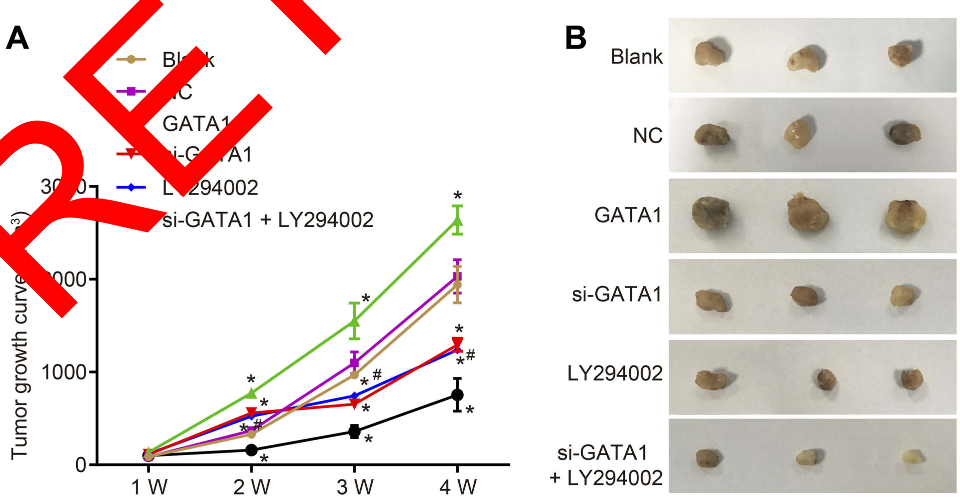



**Figure 6** siRNA-mediated downregulation of GATA1 impedes tumor initiation and tumor growth in vivo. (**A**) Tumor growth curve of the xenograft tumor of nude mice injected with GATA1, si-GATA1, LY294002 and si-GATA1+LY294002; (**B**) representative images of tumors of nude mice injected with GATA1, si-GATA1, LY294002 and si-GATA1+LY294002 at 28th day. *$p<0.05$ vs the blank and NC groups; #$p<0.05$ vs the si-GATA1 and LY294002 groups. Measurement data were expressed as means±standard deviation. The count data were expressed as a percentage or a ratio. Comparison between multiple groups at different time points using repeated measures ANOVA.

## siRNA-mediated downregulation of GATA1 inhibits the self-renewal ability of CCA stem cells

The effects of GATA1 on CCA stem cell self-renewal ability were investigated by means of spheroids formation assay. As presented in Figure 7A and B, compared with the blank and NC groups, the number and volume of spheres in the GATA1 group significantly increased ($p$<0.05), while the si-GATA1, LY294002 and si-GATA1+LY294002 groups showed significantly decreased number and volume of spheres ($p$<0.05). No statistical difference was evident between the si-GATA1 and LY294002 groups ($p$>0.05). In comparison with the si-GATA1 and LY294002 groups, the si-GATA1+LY294002 group displayed significantly decreased number and volume of spheres ($p$<0.05). Together, these data supported the conclusion that downregulated GATA1 and inactivated PI3K/AKT pathway inhibited the self-renewal ability of CCA stem cells.

## Discussion

CCA arises in the epithelium lining the bile ducts and usually cannot be diagnosed until advanced stages due to the lack of early clinical symptoms and biomarkers.[29] Surgical intervention is the only research supported modality for the treatment of CCA.[30] In order to investigate the molecular mechanism by which GATA1 was implicated in CCA, the biological characteristics of CCA stem cells were evaluated after siRNA-mediated GATA1 silencing by means of the PI3K/AKT pathway. Collectively, the data of our study revealed that downregulation of GATA1 could impede the proliferation, self-renewal,

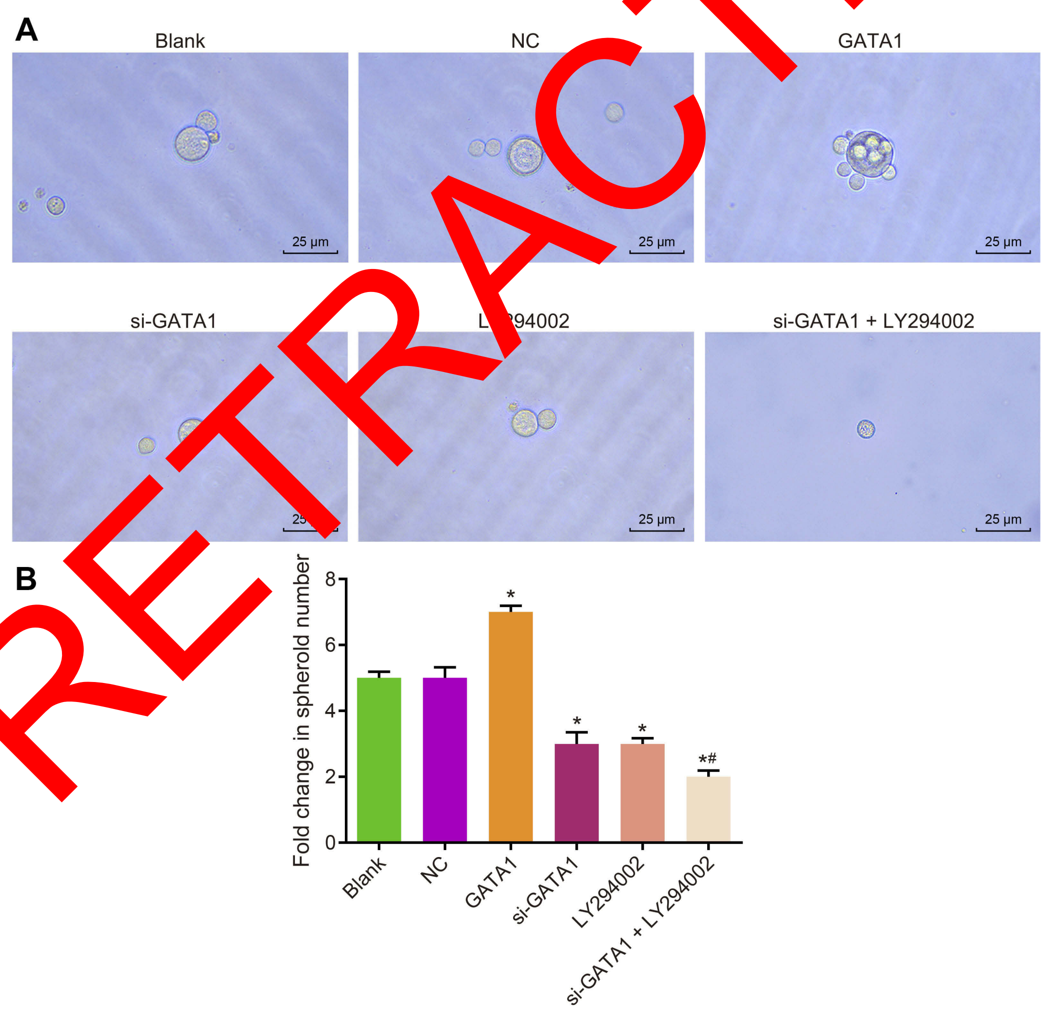



**Figure 7** siRNA-mediated downregulation of GATA1 represses the self-renewal ability of CCA stem cells. (**A**) The number and volume of spheres after transfection with GATA1, si-GATA1, LY294002 and si-GATA1+LY294002 assessed by spheroid formation assay. (**B**) Statistics regarding the number and volume of spheres after transfection with GATA1, si-GATA1, LY294002 and si-GATA1+LY294002. *$p$<0.05 vs the blank and NC groups; #$p$<0.05 vs the si-GATA1 and LY294002 groups.

migration and invasion of tumor stem cells while simultaneously promoting cell apoptosis as well as the primary cilium expression, and tumor growth via blockade of the PI3K/AKT pathway.

Our findings illustrated that the CCA stem cells transfected with si-GATA1 and si-GATA1+LY294002 exhibited decreased expression of GATA1, PI3K, AKT and mTOR proteins and reduced extent of AKT phosphorylation, suggesting that GATA1 silencing impeded the functioning of the PI3K/AKT pathway in CCA. Research has highlighted the functionality of AKT as a crucial downstream kinase of PI3K, with evidence supporting the role of activated AKT pathway as the main mediator for cell angiogenesis, growth, proliferation and survival in various cancers including lung, breast and gastric cancers.[24] An existing study provided evidence supporting the role of the AKT pathway in the progression of CCA.[31] A correlation between the reduced expression of GATA1 with advanced tumor disease and higher risk of disease recurrence has been speculated by a former study.[32] We employed the STRING database to perform protein interaction analysis in the CCA-related genes and ascertained that GATA1 directly interacted with PIK3R1, thereby affecting CCA-related genes. The aforementioned literature supports the speculation that GATA1 may play a role in the PI3K/AKT pathway and CCA progression.

Additionally, our results found that siRNA-mediated downregulation of GATA1 decreased the proliferation rate of CCA stem cells by successful inhibition of the PI3K/AKT pathway. A recent study provided evidence demonstrating that the PI3K inhibitor, LY294002, impedes the proliferation of bladder cancer cells.[33] Research supports the functionality of LY294002 as an inhibitor of the growth of cancer cells by inducing cell apoptosis and cell cycle arrest, and thus serves as a promising therapeutic compound.[34] We obtained evidence highlighting the existence of diminished migration and invasion ability of CCA stem cells upon GATA1 gene silencing and PI3K/AKT pathway inactivation. Partly in line with our findings, existing literature reported that LY294002 suppresses the migration and invasion abilities of leukemia cells[35] as well as decreases the capacity of hepatocarcinoma cell migration and invasion.[36] Moreover, we found that the apoptosis rates of CCA stem cells treated with GATA1 silencing and PI3K/AKT pathway inactivation were significantly increased. A former research demonstrated the association of LY294002 with apoptosis of human nasopharyngeal carcinoma,[37] and other studies also revealed that LY294002 is capable of inducing apoptosis in HL-60 cells[38] and promoting cell apoptosis caused by melatonin.[39] It was also reported that osthole promotes apoptosis while impeding proliferation through the PI3K/AKT pathway in intrahepatic CCA.[24] The above findings suggest that GATA1 silencing and PI3K/AKT pathway blockade inhibit the proliferation, invasion and migration while inducing apoptosis of CCA stem cells.

Furthermore, the current study illustrated that CCA stem cells transfected with si-GATA1+LY294002 exhibited increased primary cilium expression. As an organelle that consists of a unique membrane component of proteins and lipids, primary cilium regulates a great variety of signaling functions including cell migration, autophagy and decreased intercellular interaction.[40] In addition, primary cilium has been reported to serve as a therapeutic target in CCA.[41] Besides, primary cilia dysfunction has been previously observed in CCA and the loss of cilia is related to the dysregulation of several molecular pathways caused as a result of CCA development and progression.[42] It is also noteworthy that primary cilia play a key role in tumorigenesis and tumor progression by functioning as a tumor suppressor organelle.[41] Moreover, the results of our experiment also presented a lower proportion of tumors and tumor stem cells. Furthermore, our experiment demonstrated that the volume of tumor was reduced as a result of GATA1 gene silencing, highlighting that silencing of GATA1 could impede the growth of tumor via blockade of the PI3K/AKT pathway.

## Conclusion

In summary, the key findings of the current study suggest that GATA1 gene silencing contributes to the inhibition of CCA stem cell invasion, proliferation and migration through hindering the activation of the PI3K/AKT pathway. These findings provide a new insight with a novel therapeutic target for CCA treatment. Yet, owing to the limitation of time and expenditure, we have not conducted the tumor formation in nude mice with an enlarged sample size, and silenced or overexpressed GATA1 in the parental cells. Our further study will try to overcome these limitations so as to achieve more convincing results. Moreover, the specific mechanism of GATA1 is not fully elucidated, and therefore, further large-scale studies are required to illustrate the underlying mechanism.

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## Disclosure

The authors report no conflicts of interest in this work.

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## Supplementary materials

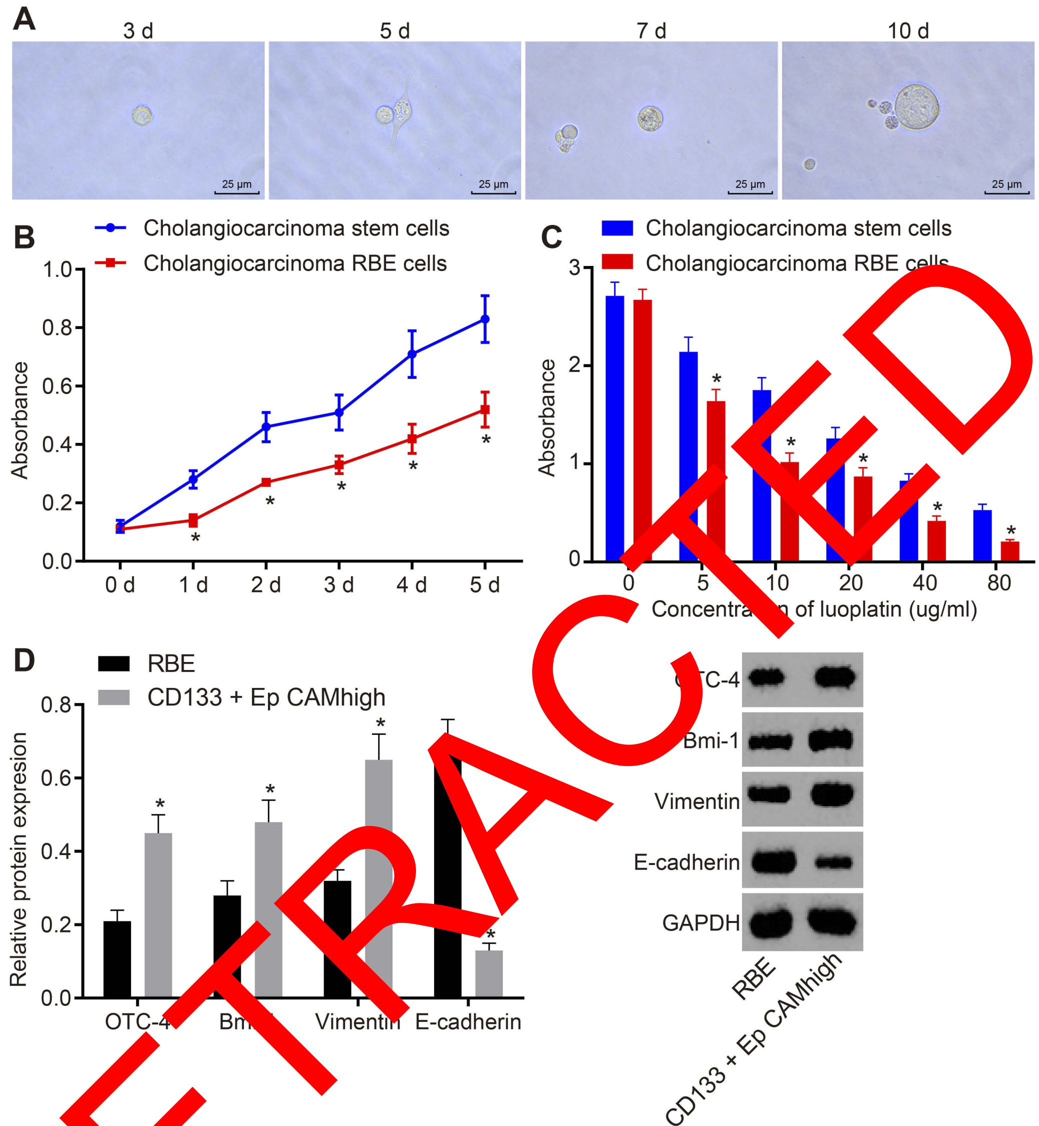




**Figure S1** CAM$^{high}$ stem-like cells from RBE cells show elevated monoclonal formation rate, OD value and tumor formation rate. (**A**) Monoclonal formation images of CD133+Ep CAM$^{high}$ stem-like cells from RBE cells; (**B**) OD values of CD133+Ep CAM$^{high}$ stem-like cells from RBE cells; (**C**) OD values of CD133+Ep CAM$^{high}$ stem-like cells from RBE cells when the concentration of Lobaplatin was the same; (**D**) protein expression of CD133+Ep CAM$^{high}$ stem-like cells from RBE cell-related nuclear transcription factors. *$P<0.05$ vs RBE cells. Measurement data were expressed as means±standard deviation. The count data were expressed as a percentage or a ratio. Comparison between multiple groups at different time points was conducted using repeated measures ANOVA. Comparisons between two groups were analyzed by independent sample *t*-test.

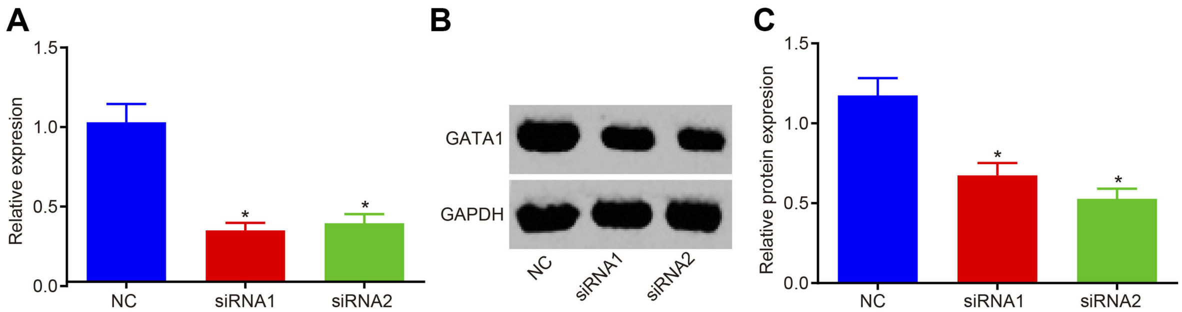


**Figure S2** siRNA1 and siRNA2 successfully silenced GATA1. (**A**) The mRNA expression of GATA1 in stem cells from RBE cells in response to NC, siRNA1 and siRNA2 measured by RT-qPCR; (**B** and **C**) the protein bands and expression of GATA1 in stem cells from RBE cells in response to NC, siRNA1 and siRNA2 measured by Western blot analysis. *$p$<0.05 vs the NC group. Measurement data were expressed as means±standard deviation. Comparisons among multiple gro ssessed by one-way ANOVA followed by Tukey post hoc test.

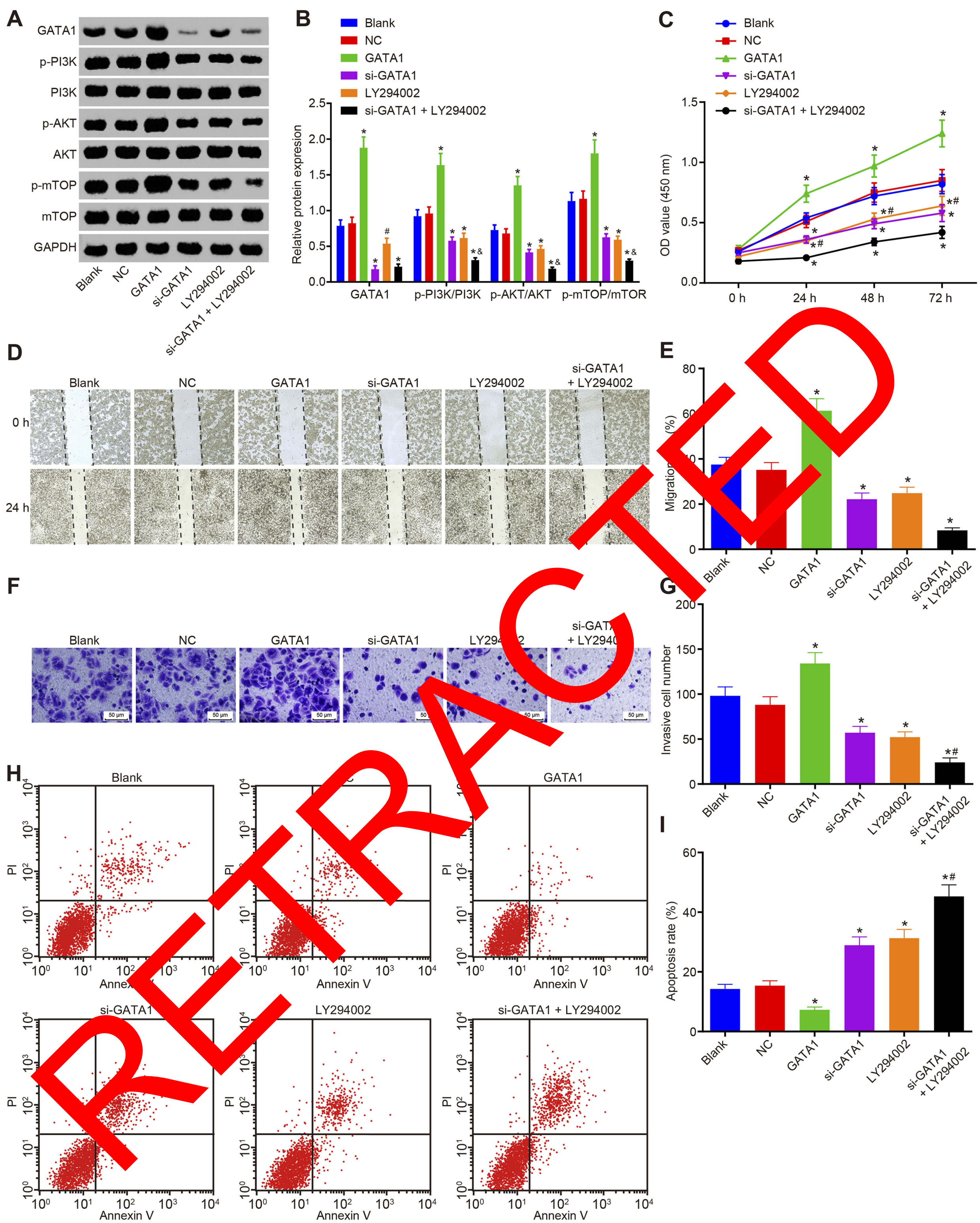


**Figure S3** siRNA-mediated downregulation of GATA1 represses invasion, proliferation and migration and accelerates apoptosis of CCA stem cells via blockade of the PI3K/AKT pathway. Stem cells from RBE cells were treated with GATA1, si-GATA1, LY294002, si-GATA1+LY294002. (**A** and **B**) Protein bands and expression of GATA1 as well as the extent of PI3K, AKT and mTOR phosphorylation in stem cells from RBE cells measured by Western blot analysis; (**C**) cell proliferation rate at 24 hrs, 48 hrs and 72 hrs in stem cells from RBE cells assessed by CCK-8; (**D-E**) cell migration ability of stem cells from RBE cells detected by scratch test (×40); (**F**–**G**) cell invasion ability of stem cells from RBE cells evaluated by Transwell assay (×200); (**H** and **I**), cell apoptosis of stem cells from RBE cells examined by Annexin V/PI double staining; *$p$<0.05 vs the blank and NC groups; #$p$<0.05 vs the si-GATA1 group; &$p$<0.05 vs the LY294002 group. Measurement data were expressed as means±standard deviation. Comparisons between multiple groups were assessed by one-way ANOVA followed by Tukey post hoc test.

RETRACTED