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Effects of silencing annexin A5 on proliferation and invasion of human cholangiocarcinoma cell line

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Abstract. – OBJECTIVE: We investigated the expression of annexin A5 (ANXA5) in human cholangiocarcinoma (CCA) cell line and its effect on proliferation, migration, and apoptosis of human CCA cells.

MATERIALS AND METHODS: Expression of ANXA5 was detected by fluorescent quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and western blotting method in 2 human CCA cell lines, QBC939 and RBE. 3 shRNA plasmids for ANXA5 silencing (ANXA5-sh1, ANXA5-sh2, ANXA5-sh3) and 1 negative control plasmid were constructed to infect QBC939 cells. The infection efficiency, expression of ANXA5, apoptosis and cell cycle of QBC939 cell were measured separately.

RESULTS: The expression of ANXA5 in QBC939 cell was significantly higher than RBE cell. Expressed ANXA5 protein in the QBC939-KD cell (QBC939 cell treated by RNAi) was significantly lower than QBC939-BC (QBC939 cell) and QBC939-NC cells (QBC939 cell treated by scramble plasmid). The ratio of G0/1 phase cells and apoptosis rate increased in QBC939-KD cell. The proliferation activity and invasion ability decreased in QBC939-KD cell compared with QBC939-NC and QBC939-BC cells.

CONCLUSIONS: ANXA5 play important role in the migration and apoptosis of CCA cells. Inhibiting the expression of ANXA5 significantly reduce the proliferation, migration and invasion ability of QBC939 cells, and increase the apoptosis of QBC939 cells.

Key Words
Annexin A5, RNAi, Bile duct carcinoma, Cell, Apoptosis.

Introduction

Cholangiocarcinoma (CCA) or bile duct carcinoma is a malignant tumor originated from bile duct epithelial cells. According to the anatomical location of the tumor, CCA is divided into extrahepatic bile duct carcinoma and intrahepatic cholangiocarcinoma[1]. Compared with intrahepatic cholangiocarcinoma, extrahepatic bile duct carcinoma is more common in CCA patients[2]. Ranked after liver cancer, CCA accounts for about 3% of gastrointestinal cancer worldwide[3] and accounts for 10% to 15% of malignant hepatobiliary tumor[4]. The incidence rate of CCA increased gradually in recent years. Northeastern Thailand and China have the highest incidence rate of CCA (96/100,000), and the lowest incidence area of CCA is Australia (0.2/100,000)[5]. The anatomical and physiological characteristics made early diagnosis and treatment of CCA difficult. The clinical symptoms of CCA at early stage is latent, occurrence of typical symptoms often means CCA already entered into the advanced stage, and the cure rate is very low[6].

Surgery is the effective treatment for CCA only when CCA is diagnosed at the early stages. CCA is not sensitive to chemotherapy, and recurrence often occurred within 2 years after surgery. The 5-year survival rate of CCA is about 5%-15%[7,8]. Reports[9,10] showed over 50% CCA patients can only receive palliative treatment due to metastasis or progression of CCA. The invasion and metastasis of CCA are the key factors affecting patients' survival. annexin A5 (ANXA5) a Ca^{2+} regulated phospholipid membrane binding protein of the annexin family[11,12]. ANXA5 mainly exists in the cytoplasm; it can be secreted outside the cell. ANXA5 participates in a variety of biological and metabolic activities, including anticoagulation[13], exocytosis, endocytosis, cell proliferation[14], signal transduction[15], and cell apoptosis[16]. Abnormal expressed ANXA5 is associated with many diseases[17,18].

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It is interesting that ANXA5 plays the opposite role in different tumors. Expression of ANXA5 is positively correlated with advance of colon cancer[18]. And it promotes invasion and chemo-resistance to temozolomide in glioblastoma cells[19]. On the other hand, ANXA5 inhibits expression of protein kinase C (PKC) directly, and finally promote the apoptosis of tumor cells[20]. The role of ANXA5 in cholangiocarcinoma is not well elucidated. This study investigated the expression of ANXA5 in human CCA cells, and the effect of ANXA5 on the proliferation, apoptosis, migration and invasion of QBC939 cells.

Materials and Methods

Cell line

Two human CCA cell lines, QBC939 and RBE, were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, CHN). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Life Technologies, Grand Island, NY, USA) (containing 10% fetal bovine serum and 100 U/L of penicillin and streptomycin) then sub-cultured in 37°C incubator with 5% CO_2.

Expression of ANXA5 in human cholangiocarcinoma cell line

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect ANXA5 mRNA expression. QBC939 cells and RBE cells in logarithmic phase were collected and washed with cold PBS buffer. The total RNAs was extracted by RNA Isolation Kit (Vazyme Biotech, Nanjing, China). cDNA was reverse transcripted. Human glyceraldehyde -3- phosphate dehydrogenase (GAPDH) was chosen as the internal reference gene. SYBR Green I (qPCR SYBR-Green Master Mix, Vazyme Biotech, Nanjing, CHN) was used as fluorescent dye. Used primers for amplification (designed and synthesized by Gene Chem Biotech, Shanghai, China) were: ANXA5: (F) 5'-AACCCTCTCGGCTTTATGATGC-3', (R) 3'-CGCTGGTAGTACCCTGAAGTG-5'. GAPDH (F) 5'- TGACTTCAACAGCGACACCCA-3', (R) 3'-CACCCTGTTGCTGTAGCCAAA-5'. Mx3000P fluorescent quantitative PCR instrument was used for PCR reaction (Agilent, Santa Clara, CA, USA). The relative expression of ANXA5 was calculated by $2^{-\Delta Ct}$ method ($\Delta Ct = CT_{ANXA5} - CT_{GAPDH}$). Protein expression of ANXA5 was measured by Western blotting. Briefly, cell lysate was added to cells, total proteins were collected, protein concentration was determined by bicinchoninic acid (BCA) assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%, 25 μg/ well); then, they were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Life Science, Boston, MA, USA). The membrane was cultured in skimmed milk at 4°C overnight. The rabbit anti-human ANXA5 monoclonal antibody (1:300 diluted) or mouse anti-human GAPDH monoclonal antibody (1:2,000 diluted) were added and cultured 1 hour at room temperature; then, a fluorescent dye-labeled secondary antibodies (1:5000) were added and cultured 2 hours at room temperature. The Odyssey® Imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used for semi-quantitative analysis (Quantity One image analysis system, BioRad Laboratories, Inc., Hercules, CA, USA).

Construction of lentiviral vector for RNA interference

GV248 plasmid (TIANGEN Biotech, Beijing, CHN) was used to construct RNAi lentiviral vector. The vector was digested to linear form by restriction endonuclease enzyme Age I (ACCGGT) and EcoR I (GAATTC). Targets RNAi sequences (Table I) were connected to the linear GV248 vector. Scramble sequence (TTCTCCGAACGTGTCACGT) was used in control group. According to the sequence of targets gene, the single oligomeric chain of DNAs (Table II) for shRNA was synthesized (Gene Chem Biotech, Shanghai, CHN). The double stranded shRNA oligonucleotide was obtained, digested and linked in GV248 vector. The recombined plasmids were named as psc-anxa5-sh1, psc-anxa5-sh2, and psc-anxa5-sh3 separately. Recombined plasmids were transformed into competent E. Coli DH5 alpha cell. Single colonies were cultured in flask. The positive clone was identified by sequencing. The successfully constructed plasmids were extracted.

Table I. shRNA targets for the ANXA5 gene.

No.	Target sequence	Start pos.
1	TGGAATTGATGAAGCTCAA	685
2	ATGATTAAGGGAGATACAT	1085
3	CGAGACTTCTGGCAATTTA	871

Abbreviation: ANXA5, Annexin A5.

Table II. Sequences of the oligo DNA for shRNA.

Oligo	Sequence of the oligomeric single-stranded DNA (5′ to 3′)
ANXA5-sh1-a	CCGGGCTGGAATTGATGAAGCTCAACTCGAGTTGAGCTTCATCAATTCCAGCTTTTTG
ANXA5-sh1-b	AATTCAAAAAGCTGGAATTGATGAAGCTCAACTCGAGTTGAGCTTCATCAATTCCAGC
ANXA5-sh2-a	CCGGCCATGATTAAGGGAGATACATCTCGAGATGTATCTCCCTTAATCATGGTTTTTG
ANXA5-sh2-b	AATTCAAAAACCATGATTAAGGGAGATACATCTCGAGATGTATCTCCCTTAATCATGG
ANXA5-sh3-a	CCGGCGCGAGACTTCTGGCAATTTACTCGAGTAAATTGCCAGAAGTCTCGCGTTTTTG
ANXA5-sh3-b	AATTCAAAAACGCGAGACTTCTGGCAATTTACTCGAGTAAATTGCCAGAAGTCTCGCG

Cell infection

After 293T cell, a lentiviral packaging cell was co-transfected by the recombined plasmid, virus packaging plasmids 1 (Helper 1) and virus packaging helper plasmid 2 (Helper 2). The virus was harvested 48-72 hours after transfection and named as LVpsc-sh1, LVpsc-sh2, LVpsc-sh3. Viruses were concentrated and purified with high titer, then preserved. The expression of green fluorescent protein (GFP) was detected under an inverted fluorescence microscope. CCA cells in exponential growth phase were prepared to 5×10^4/ml cell suspension. 4 ml of cell suspension was inoculated into 12-well plate. Enhanced infection solution (ENi.S), and polybrene were added to enhance infection. The virus multiplicity of infection (MOI, 15) was determined by pre-experiment. CCA cells were collected in 1.5 ml EP tube 16 hours after infection; then, they were centrifuged at 2000 rpm 2 min; the supernatant was removed. The rest part was mix gently and cultured in plate. 48 hours after infection, cells were treated by Puromycin (2.00 ug/ml) and observed. The efficiency of infection was measured 96 hours after infection. The most cytoplasm of infected cells showed green fluorescence under the fluorescent microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany). 4 random fields were selected from each group to analyze infection efficiency by Image Pro Plus software (repeated 3 times). If the infection efficiency were over 80%, the following research could proceed.

Expression of ANXA5 in infected CCA cells

The infected cells were collected 96 hours after infection. The expression of ANXA5 at mRNA level was measured by method described above. The silencing effect of each virus on ANXA5 gene was evaluated. The cell line (such as QBC939) containing virus with best interference effect was named as QBC939-KD (KD group), cell with packaged negative control virus (LVpsc-scramble plasmid) was named as QBC939-NC (NC group); the unaffected QBC939 cell was named as QBC939-BC (BC group). The protein expression of ANXA5 in each group was measured by the same method described above.

Effect of RNAi on the cell cycle and cell apoptosis

Flow cytometry was used to evaluate the effect of RNAi on cell cycle. Briefly, logarithmic growth cells were collected, washed with cold PBS, and fixed with 75% ethanol for 1 hours at 4°C. Then, washed with PBS, cells were adjusted to concentration of 1×10^7/ml. 1 ml iodide aziridine was added in 100 ml cell suspension, cultured at 4°C for 30 minutes then detected by flow cytometry. MultiCycle AV software (Beckman Coulter, Miami, FL, USA) was used for cell cycle analysis. Cell apoptosis was also detected by flow cytometry (Epics-XL, Beckman Coulter, Miami, FL, USA). 100 µl cell suspension (concentration of 1×10^7/ml) was suspended in 100 µl 1× binding buffer. 10 µl ANXV-APC (BD Biosciences, Franklin Lakes, NJ, USA) was added, and the suspension was cultured in the dark for 15 minutes. The cells fluorescence and the apoptosis rate in 3 groups were measured.

Effect of RNAi on migration ability of QBC939 cells

Cell migration ability was evaluated by *in vitro* wound healing model. A horizontal line was drawn evenly in the 6-well culture plate with marker-line distance of 0.5 cm. 5 lines were drawn in each well. 5×10^5 cells were inoculated in each well and reached to 100% fusion after cultured overnight. 10 µl of pipette tip was used to scratch cells along the drawn line on the bottom. The non-attached cells were removed by PBS washing. Cells were cultured in 37°C incubator with 5% CO_2. Cell images were acquired at 0 hours and 24 hours respectively.

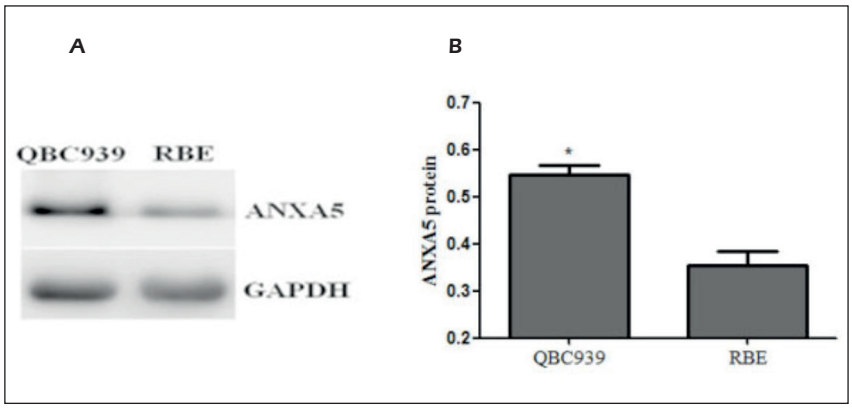

Figure 1. Assessment of the expression of ANXA5 protein in the QBC939 and RBE cells using western blotting. **A**, Western blotting showed that the expression of ANXA5 protein was higher in the QBC939 cells than that in the RBE cells. **B**, Expression of ANXA5 protein in the QBC939 vs. RBE cells (*p<0.01).

Effect of RNAi on invasion of QBC939 cells

Trans-well assay was used to evaluate invasion ability. The Extra Cellular Matrix (ECM) gel (Sigma-Aldrich, St. Louis, MO, USA) was mixed with serum-free RPMI-1640 medium (1:8). 30 µl of mixed medium was added to each Transwell chamber (Transwell®, Corning Life Science, Tewksbury, MA, USA). ECM gel was added to surface of the chamber evenly. The chamber was placed in an incubator at 37°C (with 5% CO_2) for 4 hours. After removal of the residual water in the culture medium, 50 µl RPMI-1640 serum-free medium was added to each well. Cells were cultured in 37°C incubator with 5% CO_2 for 30 minutes. Cells in logarithmic phase were collected and starved in serum-free medium for 12 hours. Then 200 µl cell suspension (containing 1×10^5 cells) were added into the upper chamber, and 600 µl complete medium were added in the lower chamber. The cells were cultured at 37°C for 24 hours. Unmoved cells in the upper chamber were wiped out gently. The moved cells were dyed by 2-3 drops of crystal violet solution about 3-5 minutes. The chamber was washed several times and dried in the air. 3 visual fields were selected randomly. The number of cells moved from the upper chamber to lower chamber was counted under a microscope at 200× magnification.

Statistical Analysis

SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data are presented as the mean ± standard deviation (SD). Analysis of variance (Bonferroni adjustment for post-hoc analysis) and independent t-tests were performed for group comparison. p<0.05 was considered as a statistically significant difference.

Results

Expression of ANXA5 in human cholangiocarcinoma cell lines

Fluorescent quantitative RT-PCR and western blotting were used to measure ANXA5 expression in 2 human cholangiocarcinoma cells, QBC939 and RBE. The relative expression of ANXA5 mRNA was 0.0629 ± 0.0036 in QBC939 cell and 0.0227 ± 0.0028 in RBE cell (p <0.01). The relative expression of ANXA5 protein (Figure 1) was 0.5467 ± 0.0351 in QBC939 cell and 0.3533 ± 0.0551 in RBE cell (p <0.01). Thus, QBC939 cell was used for following studies.

Constructed RNAi lentiviral vector

The sequencing results (Figure 2) showed the lentiviral vectors for RNAi were constructed successfully. The tested sequences were identical to designed sequences (in Supplementary Table, the shaded part shows shRNA primers, and insert sequences are in box). After packaging and transfection of plasmid to 293T cells, 3 viruses (LVpsc-sh1/2/3) were harvest. The virus titer was calculated according to the formula (virus titer = number of fluorescent cells / the amount of virus solution). The calculated virus titer of LVpsc-sh1/2/3 was 8E + 8 TU/ml according to related fluorescence microscope images (Figure 3).

The efficiency of virus infection

4 groups of viruses, LVpsc-sh1 (KD1 group), LVpsc-sh2 (group KD2), LVpsc-sh3 (KD3 group) and negative control (LVpsc-scramble, NC) virus, were used to infect QBC939 cells. 96 hours after infection, cells with green fluorescent could be observed under a fluorescence microscopy, while blank control (BC group, QBC939 cell) has no green fluorescence (Figure 4). After calculation,

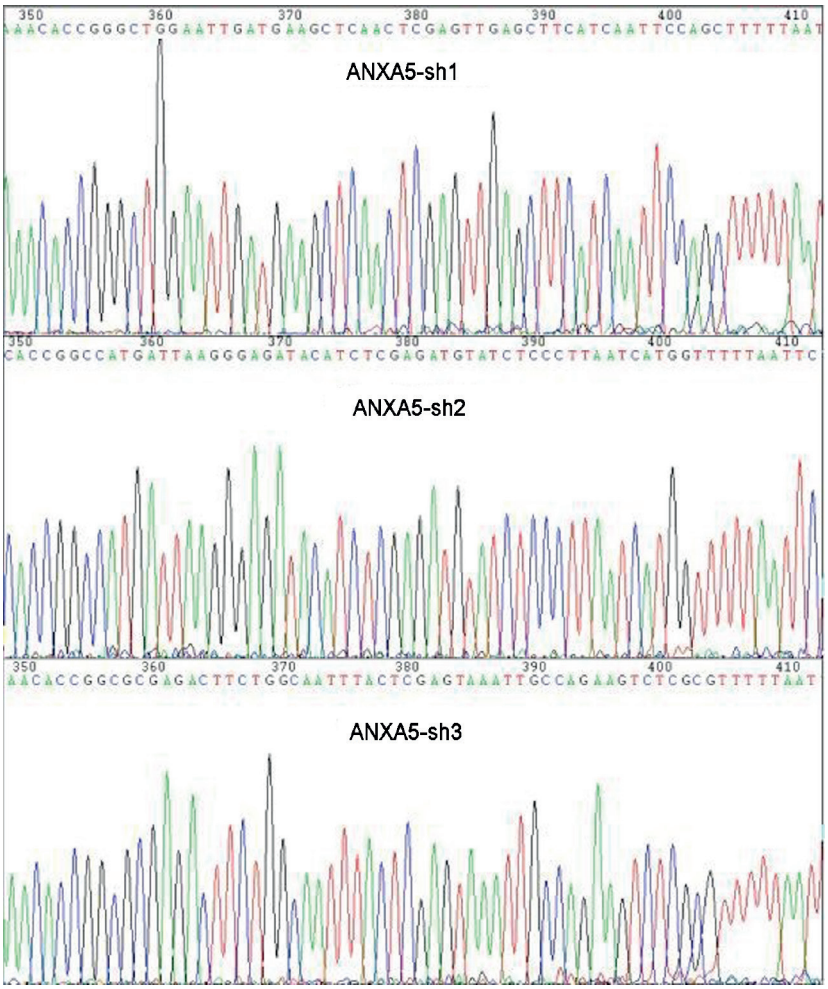

Figure 2. Sequences of the shRNA interference lentiviral vector (part).

the virus infection efficiency of KD1, KD2, KD3 and control group (NC) were 86.97± 0.45%, 82.25±1.17%, 83.33± 0.52% and 81.41± 0.39%, respectively.

Expression of ANXA5 in QBC939 cells after RNAi

qRT-PCR results showed the relative mRNA expressions of ANXA5 in LVpsc-sh1 (KD1 group), LVpsc-sh2 (KD2 group), LVpsc-sh3 (KD3 group) and LVpsc-scramble (NC group) infected QBC939 cells were: 0.0060 ± 0.0003, 0.0098 ± 0.0004, 0.0306 ± 0.0027 and 0.05487 ± 0.0059, respectively. And 0.0643 ± 0.0047 in the control group (BC group). The expression of ANXA5 mRNA in the KD1 group was significantly lower than other groups (p <0.05; Figure 5A), suggesting that the KD1 group has the best silencing effect on ANXA5 gene. Therefore, KD1 group was selected for subsequent cell function experiments.

The LVpsc-sh1 infected QBC939 cell was named as KD group then. The LVpsc-scramble infected QBC939 cell was named as negative control group (NC group); QBC939 cell was selected as blank control group (BC group). The relative expression of ANXA5 protein in the KD group (0.1546 ± 0.02151) was significantly lower than the NC group (0.5212 ± 0.01526) and the BC group (0.5612 ± 0.01237) (p <0.01; Figure 5B), suggesting that the transfection of ANXA5-targeting shRNA downregulated the expression of ANXA5 protein significantly.

The effect of RNAi on cell cycle of QBC939 cells

The flow cytometry results (Figure 6A) showed the ratio of G0/1 phase cell in the KD group (51.25 ± 1.568%) was significantly higher than the NC group (49.21 ± 1.20%) and the BC group (48.13 ± 1.392%) (p <0.05, compared with the NC and the BC group). The proliferation

Supplementary Table III. Sequence results of constructed RNAi lentiviral vector.

shRNA	Sequencing results. (shRNA primer shRNA target site)
ANXA5-sh1	AAAGAATTACAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTATT ACAGGGACAGCAGAGATCCAGTTTGGTTAATTAATCGAGCGGCCGCCCC CTTCACCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGAT ACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAA GATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAG TTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGT AACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGAC GAAACACCGGGCTGGAATTGATGAAGCTCAACTCGAGTTGAGCTTCATC AATTCCAGCTTTTTAATTCTCGACCTCGAGACAAATGGCAGTATTCATCC ACGGATCCTAACCCGTGTCGGCTCCAGATCTGGCCTCCGCGCCGGGTTT TGGCGCCTCCCGCGGGCGCCCCCCTCCTCACGGCGAGCGCTGCCACGTC AGACGAAGGGCGCAGCGAGCGTCCTGATCCTTCCGCCCGGACGCTCAG GACAGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAGTATCAG CAGAAGGACATTTTAGGACGGGACTTGGGTGACTCTAGGGCACTGGTTT TCTTTCTAGAGAGCGGAACAGGCGAGGAATAATA
ANXA5-sh2	CAGATTACAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTATTAC AGGGACAGCAGAGATCCAGTTTGGTTAATTAATCGAGCGGCCGCCCCCT TCACCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATAC AAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGA TATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTT TGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAA CTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGA AACACCGGCCATGATTAAGGGAGATACATCTCGAGATGTATCTCCCTTA ATCATGGTTTTTAATTCTCGACCTCGAGACAAATGGCAGTATTCATCCAC GGATCCTAACCCGTGTCGGCTCCAGATCTGGCCTCCGCGCCGGGTTTTG GCGCCTCCCGCGGGCGCCCCCCTCCTCACGGCGAGCGCTGCCACGTCAG ACGAAGGGCGCAGCGAGCGTCCTGATCCTTCCGCCCGGACGCTCAGGAC AGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAGTATCAGCAG AAGGACATTTTAGGACGGGACTTGGGTGACTCTAGGGCACTGGTTTTCT TTCCAGAGAGCGGAACAGGCGAGGAaAAGTAGTCCCTTCTCGGCGATTC TGCGGAGGGATCTCCGTGGGGCGGTGAACGCCGATGATTATATAAGGA CGCCGCCGGGTGAGGCACAGC
ANXA5-sh3	AAAGAATTACAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTATT ACAGGGACAGCAGAGATCCAGTTTGGTTAATTAATCGAGCGGCCGCCCC CTTCACCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGAT ACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAA GATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAG TTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGT AACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGAC GAAACACCGGCGCGAGACTTCTGGCAATTTACTCGAGTAAATTGCCAGA AGTCTCGCGTTTTTAATTCTCGACCTCGAGACAAATGGCAGTATTCATCC ACGGATCCTAACCCGTGTCGGCTCCAGATCTGGCCTCCGCGCCGGGTTT TGGCGCCTCCCGCGGGCGCCCCCCTCCTCACGGCGAGCGCTGCCACGTC AGACGAAGGGCGCAGCGAGCGTCCTGATCCTTCCGCCCGGACGCTCAG GACAGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAGTATCAG CAGAAGGACATTTTAGGACGGGACTTGGGTGACTCTAGGGCACTGGTTT TCTTTCCAGAGAGCGGAACAGGCGAGGAAAAGTAGTCCCTTCTCGGCGA TTCTGCGGAGGGATCTCCGTGGGGCGGTGAACGCCGATGATTATATAAG GACGCGCCGGGTGTGGCACAGCTAGTTTCCGTCGCAGCCGGGATTTTGG GTCGCGATTCTTGGTTT

index (PI, an index reflects the cell proliferation activity, i.e. the ratio of S and G2/M phase cells to all cells) in the KD group was significantly lower than the NC group and the BC group (p <0.05). There was no significant difference in ratio of G0/1 phase and PI between NC group and BC group (Figure 6B).

The effect of RNAi on the apoptosis rate of QBC939 cells

The apoptosis rate of KD group (12.30 ± 0.3579 %) was significantly higher than NC group (4.72 ± 0.2893%) and BC group (4.21± 0.2577%) (p <0.01). The apoptosis rate in the BC and the NC group have no significant difference (p> 0.05; Figure 7).

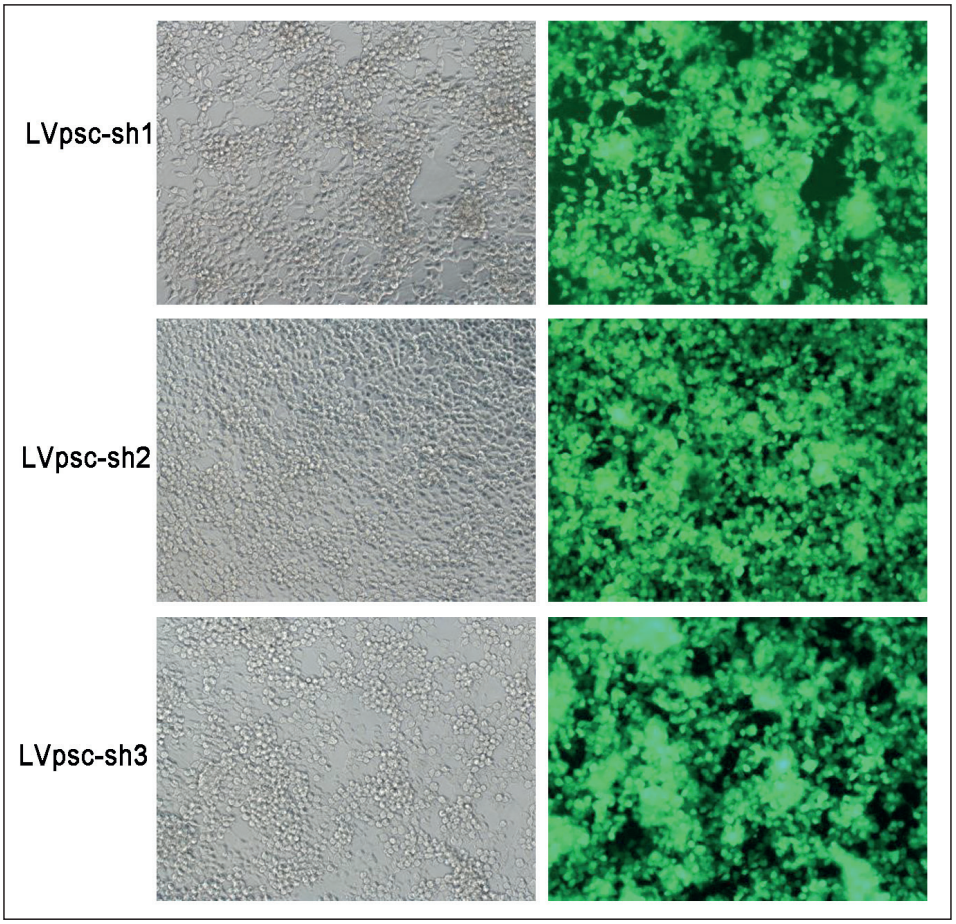

Figure 3. Images of the bright field and green fluorescent field of 293T cells infected with virus, left row images: bright field (200×); right row images: green fluorescent field (200×).

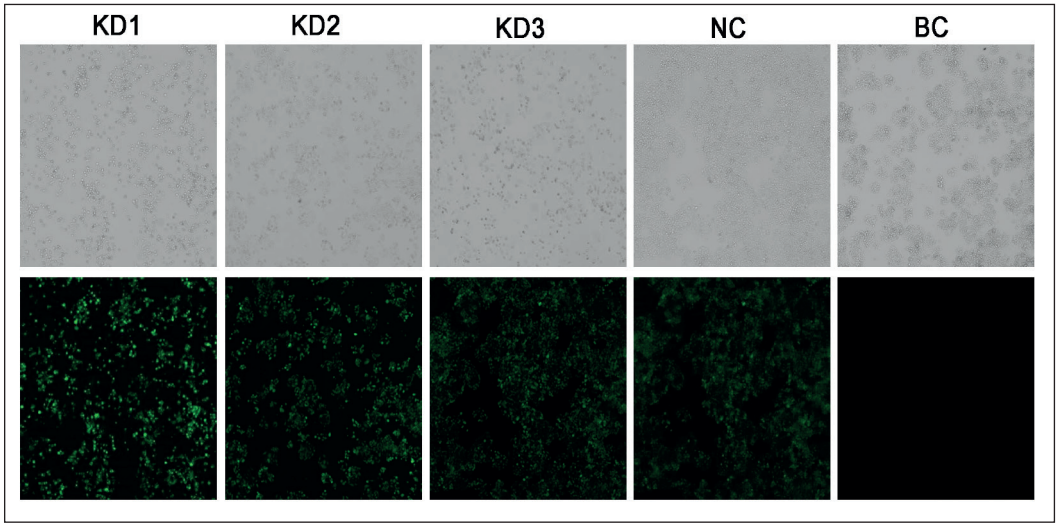

Figure 4. Observation of the expression of green fluorescence using the fluorescence microscope (magnification, 200×). KD1 group: LVpsc-sh1; KD2 group: LVpsc-sh2; KD3 group: LVpsc-sh3; NC group: LVpsc-scramble; BC group: QBC939 cell.

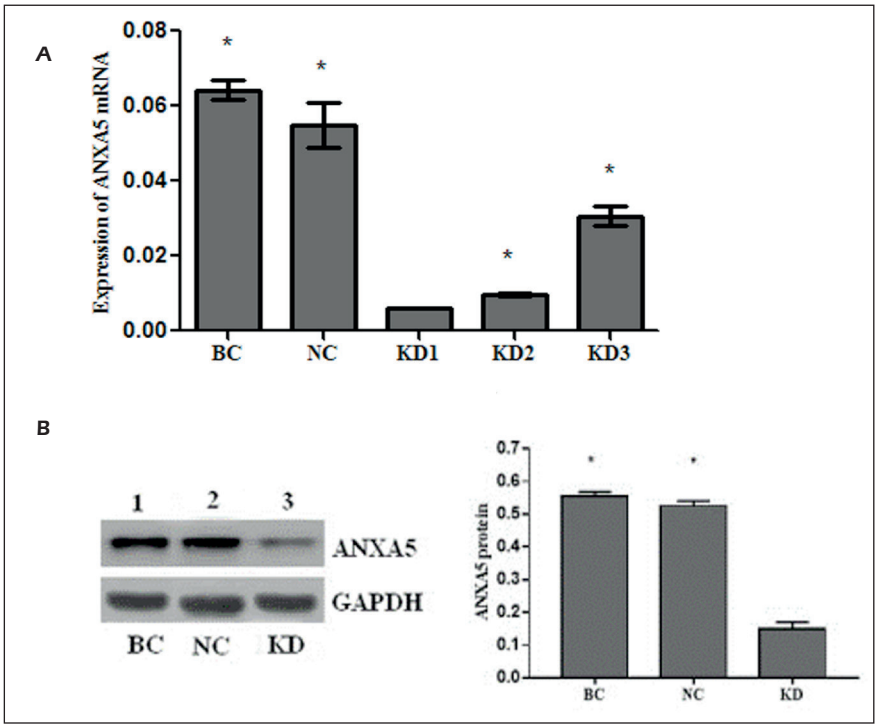

Figure 5. Assessment of the expression of ANXA5 after RNAi. **A**, mRNA expressions in the transfected cells using fluorescence quantitative RT-PCR; *p<0.05, compared with the expression of ANXA5 mRNA in the QBC939 cells transfected with LVpsc-sh1. **B**, The expression of ANXA5 protein in the transfected cells using western blotting. The expression of ANXA5 protein in the KD group cells decreased significantly, *p<0.01, compared with the BC group.

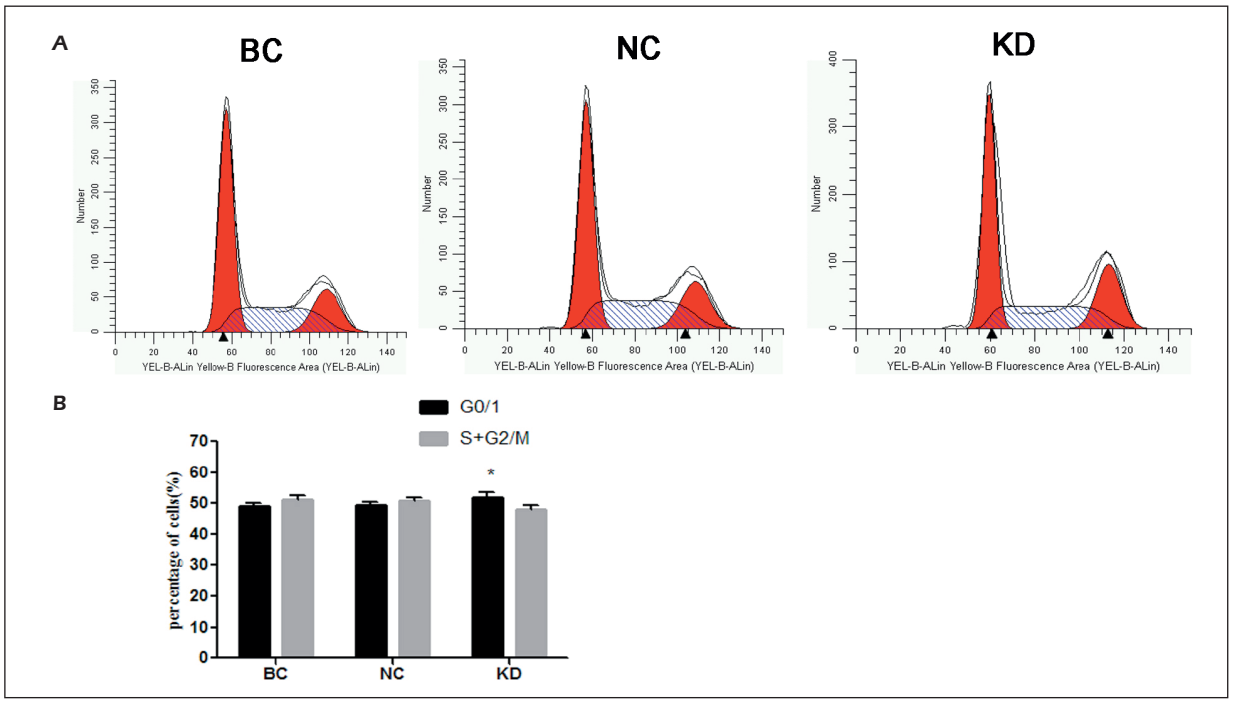

Figure 6. Evaluation of the cell cycle of the transfected cells using flow cytometry. **A**, Flow cytometry results. **B**, Bar comparison of the ratio of G0/1 phase and PI in each group. *p<0.05, compared with BC and NC groups.

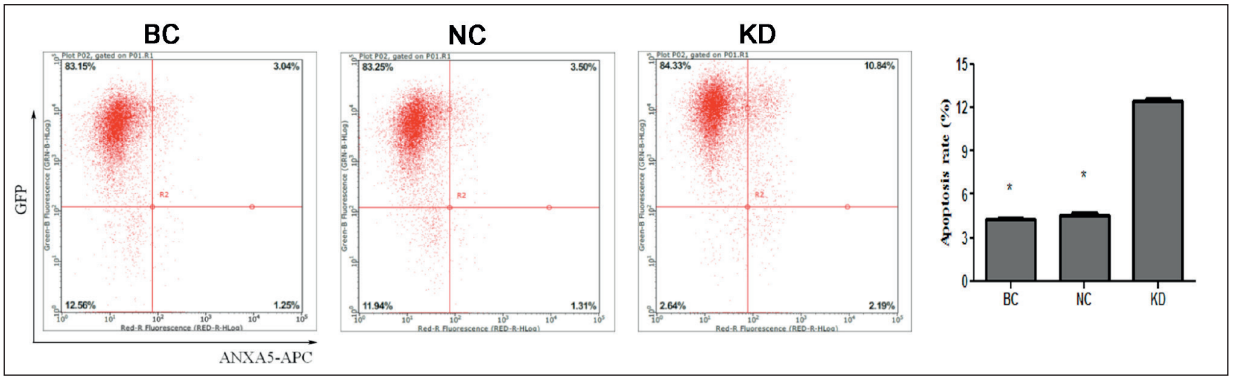

Figure 7. Evaluation of the cell apoptosis rate in blank control cells (BC), negative control cells (NC) and the transfected cells (KD) using flow cytometry. *p<0.05, compared with the NC and BC cells.

Cells' mobility changes

In vitro cell wound healing results showed that the migration ability of the KD group (0.33± 0.17 mm/24 h) were significantly lower than the NC group (0.79 ± 0.21 mm/24 h) and the BC group (0.82 ± 0.14 mm/24 h (p<0.01); Figure 8).

Cell invasion ability in Transwell

The number of cells passes through the basement membrane was 35.33± 6.03 in the KD group, which was significantly lower than the NC group (55.78 ± 8.7) and the BC group (57.65 ± 10.2) (p<0.01, Figure 9).

Discussion

Annexin A5 (ANXA5), also known as placental anticoagulant protein I, thromboplastin inhibitor V, endonexin II, calphobindin I and lipocortin V, is a member of the annexin family[21]. Its gene locates on human chromosome 4q26-q28, encoding a non-glycosylated single chain protein with 319 amino acid[22,23]. The molecular weight of ANXA5 is 35.7 KD. Researches have demonstrated ANXA5 plays an important role in the development, drug resistance and metastasis of tumors. However, the depth of research on ANXA5 cannot be comparable to other

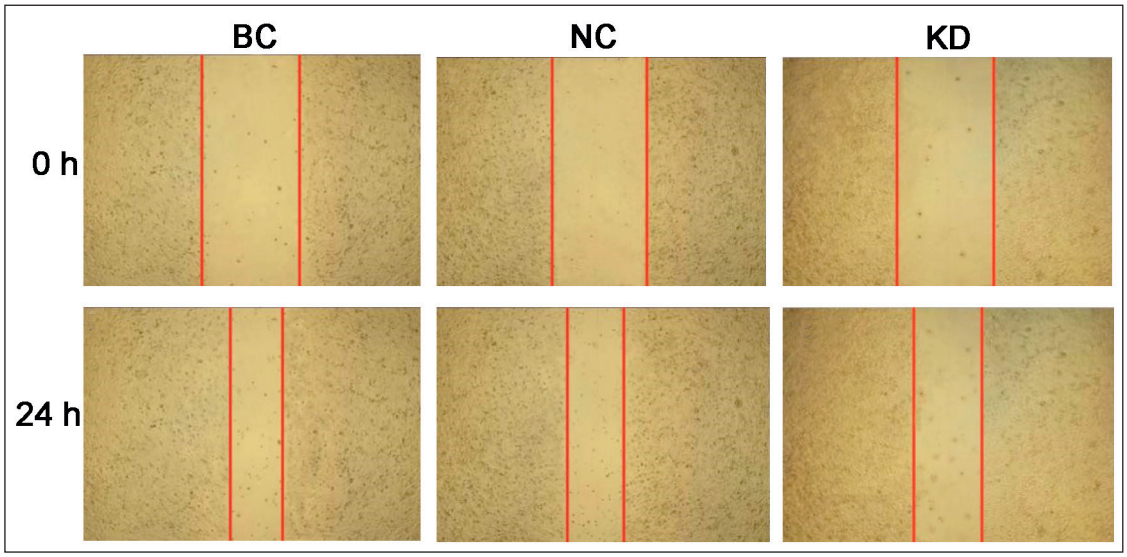

Figure 8. Evaluation of the cell migration ability using the wound healing assay (magnification, 100×).

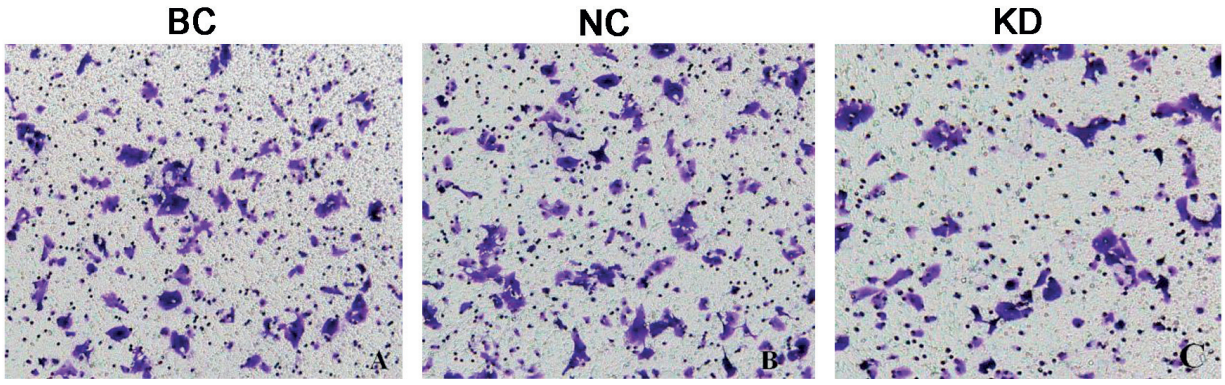

Figure 9. The cell invasion of the transfected cells under inverted microscope (magnification, 200×).

members of ANXA families, such as ANXA1, or ANXA2. In this research, we studied the effect of ANXA5 on the proliferation, apoptosis, migration and invasion ability of human cholangiocarcinoma (CCA) cell line. Our research showed that ANXA5 play important role in the migration and metastasis of CCA cells. Inhibiting the expression of ANXA5 significantly increases the apoptosis of QBC939 cells, the proliferation, migration and invasion abilities of QBC939 cells also reduced.

Using two-dimensional gel electrophoresis analyzed 5 pairs of primary liver cancer and cancer embolus samples, Guo et al[24] reported that ANXA5 was upregulated 134% in the tumor samples. They suggested that ANXA5 may become a potential biomarker for portal venous emboli. ANXA5 also related with hepatocellular carcinoma lymph node metastasis. Liu et al[25] and Sun et al[26] analyzed the lymph node metastasis potential of 2 mouse hepatoma cell lines, Hca-F and Hca-P. Results showed that lymph node metastasis potential in Hca-F was 75%, and only 25% in Hca-P. Compared with Hca-P, the expression of ANXA5 in Hca-F increased in 216%. Pancreatic ductal adenocarcinoma (PDAC) is one of the most common types of pancreatic cancer. Most PDAC are diagnosed in late stage with poor prognosis. Cui et al[27] speculated hypoxic conditions may cause up-regulation of ANXA5 in PDAC. Lacking blood supply is the imaging features of most pancreatic cancer. The insufficient blood supply may be associated with the rapid growth of tumor. Continuously exposed to severe hypoxia conditions, the hypoxia-inducible factor in cells could be activated[28]. Thus, it promotes the transcription of pro-angiogenic factors and the progress of malignant tumor; even it might increase the resistance of tumor cells to radiation and chemotherapy. Proteomics and immunohistochemical results showed that the expressed ANXA5 in pancreatic cancer nest increased by 325% compared with normal pancreatic duct. However, detailed mechanism of ANXA5 in PDAC still need further study.

Drug resistance is the main reasons for failure of chemotherapy. Compared with adjacent tissues, the multidrug resistance protein (MRP) in gastric cancer is highly expressed[29]. ANXA5 is correlated with positive expressed MRP in gastric cancer. And up-regulation of ANXA5 could promote drug resistance of gastric cancer[30]. Wu et al[31] compared the expressions of MRP and ANXA5 in 2 gastric cancer lines, SGC-7901 cell and SGC-7901/DDP cell. They indicated the expression of ANXA5 elevated in SGC-7901/DDP cell with the up-regulation of MRP protein. siRNA silencing of ANXA5 leads to decreased expression of MRP in SGC-7901/DDP cell. In addition, the IC_{50} of cisplatin, paclitaxel, and 5-Fu in SGC-7901/DDP cell decreased significantly. The sensitivity of SGC-7901/DDP cell to cisplatin, paclitaxel, and 5-Fu increased in 36, 17 and 4 times respectively. Xue et al[18] analyzed 207 pair of colorectal adenocarcinomas (CRC) and adjacent tissues. Results showed that the expression of ANXA5 increased in tumor tissues. Further analysis showed that up-regulated ANXA5 was correlated with tumor stage, liver metastasis, increased recurrence rate and low survival of these CRC patients.

Zheng et al[32] demonstrated ANXA5 affects the occurrence and development of breast cancer via Ras/Raf/MEK/ERK pathway through acting on Shc protein. This pathway plays important roles in regulating metabolism, proliferation, and differentiation of cell. Sato et al[33] and El-Shemerly et al[34] found that Shc might be the substrate

of protein kinase (PKC) in Ras/Raf/MEK/ERK signaling pathway. PKC induces phosphorylation of Shc and promotes its combination with Grb2. The overexpression of ANXA5 may inhibit Shc through inhibiting PKC. Abnormal functioned PKC lead to abnormal differentiation and proliferation of cell, and the occurrence of tumors.

ANXA5 not only plays the opposite role in different tumors[18-20], it also demonstrates the opposite roles at the cellular and organ level: ANXA5 promotes the apoptosis of colorectal cancer cells[35], on the other hand, it is highly expressed in advanced colon cancer tissues[18]. Although up-regulation of ANXA5 promotes progression of cervical cancer, it is markedly suppressed in cervical carcinoma cells. This might because ANXA5 involved in inflammatory carcinogenesis process. It mediates apoptosis of cancer cells, promotes the occurrence of immune response. After been exposure to early inflammatory cell, ANXA5 made cells with low tumorigenic and low tumorigenicity transforming into tumor cells with highly tumorigenic and metastatic. All together, these researches demonstrate ANAXA5 plays important roles in cancer; however, its mechanism is far more complex than we researched so far. A large number of follow-up studies are needed to explore and verify the related mechanisms.

Conclusions

We demonstrated that ANXA5 plays an important role in the migration and metastasis of cholangiocarcinoma cells. Inhibiting the expression of ANXA5 significantly increases the apoptosis, reduces the proliferation, migration, and invasion of QBC939 cells. Our study provides an experimental basis for the further mechanism study of ANXA5 in CCA. However, the exact mechanism needs further study.

Conflict of interest

The authors declare there is no conflict of interest.

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