Article

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Effect of Deletion of ANGPTL4 Gene on Viability, Migration and Invasion Ability and Apoptosis of Hepatocellular Carcinoma Cells

Yun Bai[1,†], Guanghua Cui[1,†], Xiaoke Sun[1], Meiqi Wei[1], Yanying Liu[1], Jialu Guo[1], Yu Yang[1,*]

[1]Department of Oncology, the Second Affiliated Hospital of Harbin Medical University, 150081 Harbin, Heilongjiang, China
*Correspondence: yangyu13836125585@126.com (Yu Yang)
[†]These authors contributed equally.
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Background: Hepatocellular carcinoma (HCC) is a malignant tumor that impacts individuals worldwide and is particularly prevalent in Asia. Angiopoietin-like protein 4 (ANGPTL4) plays an important role in regulating glucose and lipid metabolism in mouse liver. We sought to explore the effects of the ANGPTL4 gene on cell viability, migration, invasive capacity, and apoptosis of HCC cells.

Methods: The expression of ANGPTL4 in HCC and paracancerous tissues was determined by immunohistochemistry and immunofluorescence assays. The ANGPTL4 knockdown cells were established by shRNA transfection. The effect of ANGPTL4 knockdown on HepG2 and Huh7 cells was determined by Cell Count Kit-8 (CCK-8), wound healing and transwell assays. Cell apoptosis was determined by flow cytometry.

Results: The ANGPTL4 expression was dramatically enhanced in HCC tissues than in paracancerous tissues ($p < 0.001$). HCC cell lines HepG2 and Huh7 with knockdown of ANGPTL4 gene showed lower cell viability, migration, and invasion ability while inducing higher apoptosis levels than the control group ($p < 0.001$).

Conclusions: High expression of ANGPTL4 is closely related to HCC. Knockdown of ANGPTL4 significantly inhibits the proliferation of HCC cells. This study provides a rationale for the ANGPTL4 gene, a molecular marker of HCC.

Keywords: angiopoietin-like 4; hepatocellular carcinoma; bioinformatics; angiogenesis

Introduction

Hepatocellular carcinoma (HCC) is a malignancy, accounting for 80% of primary liver cancers [1]. Morbidity and mortality have constantly increased in recent years, with approximately 900,000 new primary liver cancers and 800,000 HCC deaths worldwide, mainly in Southeast Asia [2]. Surgical treatments such as liver resection or liver transplantation are effective in patients with early-stage HCC [3,4]. However, there is a lack of effective diagnosis and treatment for patients with advanced HCC [5]. Chemotherapy is the primary treatment for advanced HCC. However, HCC's high aggressiveness and resistance to chemotherapeutic drugs lead to poor prognosis [6]. Therefore, it is necessary to find new molecular markers to unravel the therapeutic effect and predict the prognosis of HCC with high sensitivity and specificity.

Angiopoietin-like protein 4 (ANGPTL4) is a member of the angiopoietin-like protein (ANGPTL) family, which is highly similar in structure to the ANG family [7]. The ANGPTL4 protein contains a highly hydrophobic signal peptide with a coiled-coil structural domain and a fibrinogen-like structural domain at the N-terminus and C-terminus, respectively [8]. Upregulation of ANGPTL4 increase tumorigenesis, invasion, anoikis resistance, and metastasis in human colon, breast, and kidney cancers [9]. In addition, ANGPTL4 inhibits vascular permeability to produce an anti-metastatic effect [10,11]. The clinical, functional role of ANGPTL4 in HCC is still unclear. A study showed that high serum ANGPTL4 protein levels are significantly associated with cirrhosis, high histologic grade, and intrahepatic metastases in HCC patients. The current study aims to investigate the effect of ANGPTL4 on the proliferation, migration, invasion, and apoptosis of HCC cells.

Materials and Methods

Materials

The human HCC cell lines HepG2 (RRID: CVCL_0027) and Huh7 (RRID: CVCL_U443) were obtained from Beina Bio (Hebei, China). The cell lines were authenticated by short tandem repeat (STR) profiling. Fetal bovine serum (FBS), penicillin, and streptomycin obtained from GIBCO (Waltham, MA, USA), a biological spectrophotometer (Nano-500) was obtained from Hangzhou Aosheng

Instrument Co Ltd (Hangzhou, China). The BCA assay kit, propidium iodide staining solution, and RIPA lysis buffer were obtained from Beyotime Biotechnology (Shanghai, China), ANGPTL4 (Boster A01147, 1:1000) was obtained from Shanghai Wei-Ao Biotechnology Co Ltd (Shanghai, China), flow cytometer was purchased from Beckman Coulter (Miami, FL, USA), Transwell was purchased from SPL (Beon-gil, Korea), 4% paraformaldehyde was purchased from Biosharp, and closed goat serum was purchased from Solaibao Bio (Beijing, China).

Subjects

The protocol of this study was approved by the Ethics Review Committee of the Second Hospital of Harbin Medical University [Committee of the Second Hospital of Harbin Medical University KY-2022-208] in accordance with the Declaration of Helsinki, and informed consent was obtained from the patients themselves or their legal guardians. Fifty HCC patients with complete clinical data from Group 2 of the surgical oncology ward of the Second Affiliated Hospital of Harbin Medical University were selected for the study. The patient information is summarized in Table 1.

Immunohistochemistry

Sections of paraffin-embedded clinical specimens were used for immunohistochemistry staining. Paraffin sections were dewaxed with xylene, rinsed with pure alcohol, and rehydrated with water. They were then placed in a lemon buffer (pH 6.0) and microwaved on high power for 3 minutes and then on low power for 10 minutes. Sections were then blocked with 3% peroxidase for 20 minutes and 10% goat serum for 30 minutes. The sections have been incubated with ANGPTL4 antibody (1:100, Boster A01147) at a dilution of 1:100 in phosphate buffered saline (PBS) overnight at 4 °C. After carefully washing away the primary antibody solution; the slides were coated with the Goat Anti-Mouse IgG (H+L) HRP (1:1000, S0002, Affinity Biosciences Ltd.) for 1 hour at 37 °C. Finally, cell nuclei were counterstained with hematoxylin.

Cell Culture

The cells were tested for no mycoplasma contamination and cultured in 10% DMEM containing 10% FBS.

Immunofluorescence Assay

The tissue microarrays were placed vertically in the slide box and baked at 60 °C for 2–3 hours to melt off the sealing wax. For immunofluorescence experiments, 10,000 cells were inoculated in 24-well plates, fixed overnight in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 20 minutes at room temperature. After washing the slide with PBS, add normal goat serum to the 24-well plate, close it at 37 °C for 30 minutes, aspirate off the closure solution, and incubate the primary antibody in an incubator for 1 hour. Wash 3 times with PBST for 3 minutes

Table 1. HCC clinical patients' data was used in this study.

Case number	Gender	Age	Disease diagnosis	TNM
15-21402	M	53	HCC-3	T1aNxM0
15-22802	M	55	HCC-3	T1aNxM0
16-11086	M	45	HCC-3	T1aNxM0
17-15752	M	64	HCC-3	T1aNxM0
18-28596	M	57	HCC-2	T1aNxM0
19-15691	M	65	HCC-3	T1aNxM0
15-05390	M	57	HCC-2	T2NxM0
15-08906	M	63	HCC-2	T2NxM0
15-11265	M	66	HCC-2	T2NxM0
15-16446	M	59	HCC-3	T2NxM0
15-19855	M	56	HCC-3	T2NxM0
15-22068	M	55	HCC-3	T2NxM0
15-27620	M	67	HCC-4	T2NxM0
16-03333	F	71	HCC-3	T2NxM0
16-07617	M	27	HCC-3	T2NxM0
16-13817	M	58	HCC-3	T2NxM0
16-13819	M	70	HCC-2	T2NxM0
16-19020	M	70	HCC-4	T2NxM0
16-20617	F	63	HCC-3	T2NxM0
16-31768	F	55	HCC-3	T2NxM0
16-34340	F	63	HCC-3	T2NxM0
17-03208	F	61	HCC-3	T2NxM0
17-07827	M	69	HCC-4	T2NxM0
17-15962	M	66	HCC-4	T2NxM0
15-12057	F	53	HCC-4	T3NxM0
15-14563	M	53	HCC-3	T3NxM0
15-15689	M	55	HCC-3	T3NxM0
15-24505	M	63	HCC-4	T3NxM0
15-32318	F	70	HCC-2	T3NxM0
16-18983	M	60	HCC-2	T3NxM0
16-29253	M	56	HCC-3	T3NxM0
17-26008	F	61	HCC-3	T3NxM0
18-14037	F	74	HCC-4	T3NxM0
18-37411	M	72	HCC-3	T3NxM0
19-32507	M	66	HCC-3	T3NxM0
19-43618	M	63	HCC-2	T3NxM0
19-49481	M	48	HCC-3	T3NxM0
20-47425	M	63	HCC-3	T3NxM0
21-03769	F	66	HCC-2	T3NxM0
17-21974	M	74	HCC-3	T3NxM1
17-23012	M	60	HCC-4	T3NxM1
18-19172	M	66	HCC-2	T4NxM0
19-20219	M	54	HCC-3	T4NxM0
20-01650	M	52	HCC-3	T4NxM0
18-01994	M	47	HCC-3	T4NxM1
18-04863	F	63	HCC-4	T4NxM1
19-09589	M	79	HCC-2	T4NxM1
19-43143	M	50	HCC-3	T4NxM1
19-53965	M	55	HCC-1	T1aN0M0
19-52316	M	48	HCC-1	T1bN0M0

HCC, Hepatocellular carcinoma; TNM, Tumor, Nodes, and Metastasis.

each time, and add diluted fluorescent secondary antibody. After washing, add DAPI dropwise and incubate for 5 minutes, protect from light, and nucleate the specimen. Using PBST for 4 times with a duration of 5 minutes each time to remove excess DAPI. Slices were sealed with a sealing solution containing an anti-fluorescence quencher, and images were obtained using a fluorescent microscope.

Construction of Stable Knockdown of ANGPTL4 in HepG2 and Huh7 Cells

Cells with steady ANGPTL4-knockdown, or control HepG2 cells, were made utilizing shRNA 5′-GGUGAGUGCAUGUAGUCAUTT-3′, 5′-AGAACAGCAGGAUCCAGCAACUCUU-3′, or scramble control arrangement 5′-UUCUCCGAACGUGUCACGUTT-3′. shRNAs were inserted into the LV3 (H1/GFP&Puro) vector, individually. The comparing plasmids were bundled utilizing lentivirus (Shanghai GenePharma, China). The stable cell line was selected by Puromycin (Sigma, St. Louis, MI, USA).

RNA Extraction and Quantitative RT-PCR (qRT-PCR)

RNA was isolated using TRIzol. RNAs were decontaminated and measured utilizing the NanoDrop 2000 Spectrophotometer. The RT reagent Pack reversely translated RNAs. qRT-PCR tests were exhausted 10 µL containing 1× SYBR Green Ace blend, cDNA (10 ng), and groundworks (upstream primers: 5′-GATGGCTCAGTGGACTTCAACC-3′, downstream primers: 5′-TGCTATGCACCTTCTCCAGACC-3′) in an Exicycle 96 Real-Time Quantitative Warm Piece, with starting hatching at 95 °C (10 min), 40 cycles at 95 °C (15 s) and 60 °C (1 min). qRT-PCR tests were performed in triplicate, the midpoints of which were normalized (by β-actin, upstream primers: 5′-CACCATTGGCAATGAGCGGTTC-3′, downstream primers: 5′-AGGTCTTTGCGGATGTCCACGT-3′), and the relative expression of AC007639.1 was calculated utilizing the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

The cells were isolated with RIPA lysis buffer for 20 minutes on ice. The BCA assay kit determined protein concentration. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes and probed with target antibodies. Primary Antibodies included beta-actin (affinity T0022, 1:1000), ANGPTL4 (Boster A01147, 1:1000), and a secondary antibody (affinity S0001, 1:5000). The protein quantitation was performed using ImageJ version 2 (National Institutes of Health, Bethesda, MD, USA).

Cell Count Kit-8 Assay

The Cell Count Kit-8 (CCK-8) test kit measured cell proliferation. Briefly, cells were seeded into 96-well plates and incubated one day in DMEM medium containing 10% bovine serum. The 10 µL of CCK-8 reagent was added to each well. After 2 hours of incubation, the absorbance at 450 nm was measured using a microplate spectrophotometer.

Cell Apoptosis Detection

Cell apoptosis was detected by Annexin V/propidium iodide (PI) double staining. Briefly, 48 hours after transfection, cells were treated with cisplatin (20 µM) for 12 hours, harvested with trypsin, washed twice with cold PBS, and then treated with 200 µL of binding buffer. Resuspended in A staining solution containing Annexin V/FITC and PI was added to the cell suspension. After 30 minutes of incubation in the dark, cells were analyzed on a FACS Gallios flow cytometer.

Wound Healing Assay

HepG2 and Huh7 cells grown in the exponential growth phase were made into cell suspensions counted, and the number of cells seeded in each well was determined by cell type and size in a pre-assay. After rinsing with PBS until no cells were in suspension, the culture medium was replaced with the serum-free medium. Three randomly selected fields were imaged with an inverted microscope at 0 and 24 hours, respectively, and the scratch width was measured using ImageJ software (version 2.0, NIH, Bethesda, MD, USA). Cell healing rate = (original wound width – current wound width)/original wound width × 100%.

Transwell Assay for Cell Invasion

Diluted Matrigengel was added vertically to the upper chamber of Transwell and spread evenly and flatly on the bottom, then incubated in an incubator (37 °C, 5% CO_2) for 3 hours to polymerize the Matrigengel into a film. Logarithmically grown HepG2 and Huh7 cells were removed, cell suspensions were prepared and counted, and preliminary experiments were performed to determine the number of cells inoculated per well, cell type, and distribution size in 6-well plates. HCC cell lines were transfected with shRNAs, and cultured in a serum-free medium for 24 hours. The cells were then transferred to the top chamber of the Transwell plate and inoculated in 200 µL of serum-free medium. Then, 0.5 mL of medium containing 10 µL of bovine serum was added to the lower chamber as a chemoattractant. Cells were incubated at 37 °C for 0–24 hours to determine invasion ability. At the end of incubation, the upper chamber was removed, and unaffected cells in the upper chamber were swabbed with cotton swabs, fixed with 4% paraformaldehyde for 5 minutes, and stained with 0.2% crystal violet. The cells were washed three times with PBS

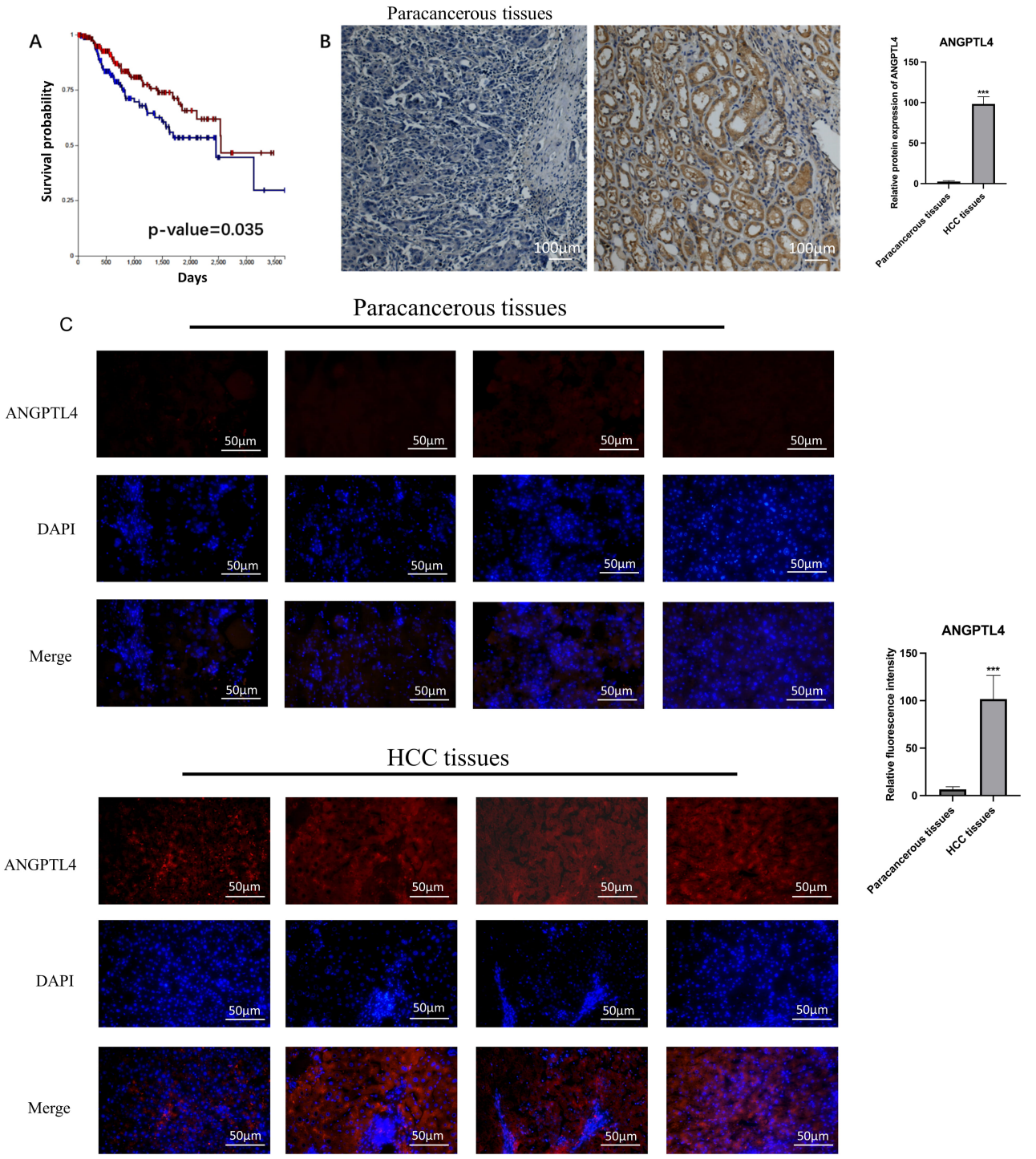

Fig. 1. ANGPTL4 is highly expressed in HCC tissues. (A) Effect of ANGPTL4 on survival of oncology patients. The blue line is the percentage of survival when ANGPTL4 is highly expressed, and the red line is the percentage of survival when ANGPTL4 is lowly expressed. (B) Expression of ANGPTL4 in HCC tissues and paracancerous tissues, ANGPTL4 protein in brown color. (C) Immunofluorescence detection of ANGPTL4 gene expression, red indicates ANGPTL4 gene. Magnifier 10×, scale bar = 50 μm. Data presented as the mean ± standard deviation. N = 4, ***$p < 0.001$. ANGPTL4, Angiopoietin-like protein 4.

at room temperature for 30 minutes, then air-dried and photographed under an inverted microscope in three randomly selected fields.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). The significance was determined by the one-way analysis of variance with Dunnett's multiple-

comparison test or *t* test. Survival curve was estimated using the Kaplan–Meier method and compared using the log-rank test. A *p* value less than 0.05 was considered as the significance level.

Results

ANGPTL4 Expression in HCC

The correlation between ANGPTL4 expression and overall survival of tumor patients was analyzed according to the TCGA-LIHC database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) using DESeq2 software (1.38.3, Bioconductor, Chicago, IL, USA). As shown in Fig. 1A, the blue line shows the percentage of survival when ANGPTL4 was highly expressed, and the red line shows the percentage of survival when ANGPTL4 was lowly expressed; the results showed that the overall survival of patients with high ANGPTL4 expression was significantly lower than that of patients with low ANGPTL4 expression ($p < 0.05$). Using an immunohistochemistry assay, the expression of ANGPTL4 in HCC tissues was significantly higher in HCC tissues than in paracancerous tissues ($p < 0.001$, Fig. 1B). The expression of ANGPTL4 was characterized by cellular immunofluorescence (IF), as shown in Fig. 1C, and the fluorescence of ANGPTL4 was significantly enhanced in cancer tissues ($p < 0.001$).

Establishment of Stable Knockdown of ANGPTL4 in HepG2 and Huh7 Cells

To explore the function of ANGPTL4 in HCC, two specific shRNAs were used to knock down ANGPTL4 levels in the HCC cell line. We tested the expression of ANGPTL4 in HepG2 and Huh7 cell lines. The results showed that knockdown of ANGPTL4 significantly reduced the relative mRNA (Fig. 2A) and protein (Fig. 2B) level of ANGPTL4 in both HepG2 cell lines and Huh7 cell lines. Both shRNA stable knockdown ANGPTL4 HepG2 cell lines showed significantly lower expression of ANGPTL4 compared to ANGPTL4-NC HepG2 cell lines ($p < 0.0001$), shRNA stable knockdown ANGPTL4-1 Huh7 cell lines showed significantly lower ANGPTL4 expression compared to ANGPTL4-NC Huh7 cell lines ($p < 0.001$), and the Huh7 cell line with shRNA stable knockdown of ANGPTL4-4 had significantly lower ANGPTL4 expression compared to the ANGPTL4-NC Huh7 cell line ($p < 0.0001$).

Deficiency of ANGPTL4 Reduces the Viability and Migratory Capacity of HCC Cells

To determine the effect of ANGPTL4 on HCC cells, we examined the cell viability and migration ability of HepG2 and Huh7 cells after knockdown of ANGPTL4. We found that shRNA-mediated knockdown of ANGPTL4 significantly down-regulated the cell viability (Fig. 3A) ($p < 0.0001$). As shown in Fig. 3B,C, the migratory ability of ANGPTL4 knockdown HepG2 and Huh7 cells was significantly reduced. The healing rate of ANGPTL4 knockdown Huh7 cells in the sh1 and sh4 groups was significantly reduced compared with that in the NC group ($p < 0.001$), and the results of ANGPTL4 knockdown HepG2 cells in the sh1 and sh4 groups were in line with those of Huh7 cells.

Decreased Invasive Capacity and Enhanced Apoptosis in HCC Cells Lacking ANGPTL4

The effect of ANGPTL4 knockdown on cell invasion ability and apoptosis was determined. As shown in Fig. 4A,B, the invasion ability of Huh7 and HepG2 cell lines with ANGPTL4 knockdown significantly decreased compared to the NC group ($p < 0.001$). As shown in Fig. 4C–F, the apoptosis ability of Huh7 and HepG2 cell lines with ANGPTL4 knockdown significantly enhanced ($p < 0.01$).

Discussion

Angiopoietin-like protein 4 (ANGPTL4) is a hepatic fibrinogen and angiopoietin-associated protein, adipose-inducible adipokine, a circulating glucose protein, and structurally belongs to the angiopoietin family [12]. ANGPTL4 has been reported to have unique biological functions, and current studies on ANGPTL4 have focused on its role in regulating lipid metabolism, especially as an inhibitor of lipoprotein lipase activity [13,14]. Although early studies detected ANGPTL4 in various organs or tissues, such as skin, intestine, kidney, adipose tissue, and liver, little is known about the relative expression of ANGPTL4 in these tissues [15]. ANGPTL4 can be stimulated under inflammatory and hypoxic conditions and also plays an important role in a variety of cancers, such as human colon, breast, and renal cancers [16], with multiple functions such as glycolipid metabolism, inflammation, differentiation, angiogenesis, and tumorigenesis [17]. There are few studies on the relationship between ANGPTL4 and HCC; we investigated the effect of ANGPTL4 on the viability, migratory and invasive capacity, and apoptotic capacity of HCC cells to provide theoretical support for the use of the ANGPTL4 gene in the non-invasive diagnosis and treatment of HCC.

Our results indicated that ANGPTL4 expression levels increased in HCC patients. ANGPTL 4 expression was significantly associated with HCC. Previous studies demonstrated overexpression of ANGPTL4 in HCC patients [18].

In studying the proliferation of the ANGPTL4 gene in HCC cells, our results showed a decrease in cell viability, migration, and invasion in HepG2 and Huh7 and an increase in the number of apoptotic cells, suggesting a possible positive effect of the ANGPTL4 gene on the growth of HCC cells, but also explaining that HCC inflammation may induce the production of the ANGPTL4 gene [19]. Studies on the role of the ANGPTL4 gene in tumor growth have

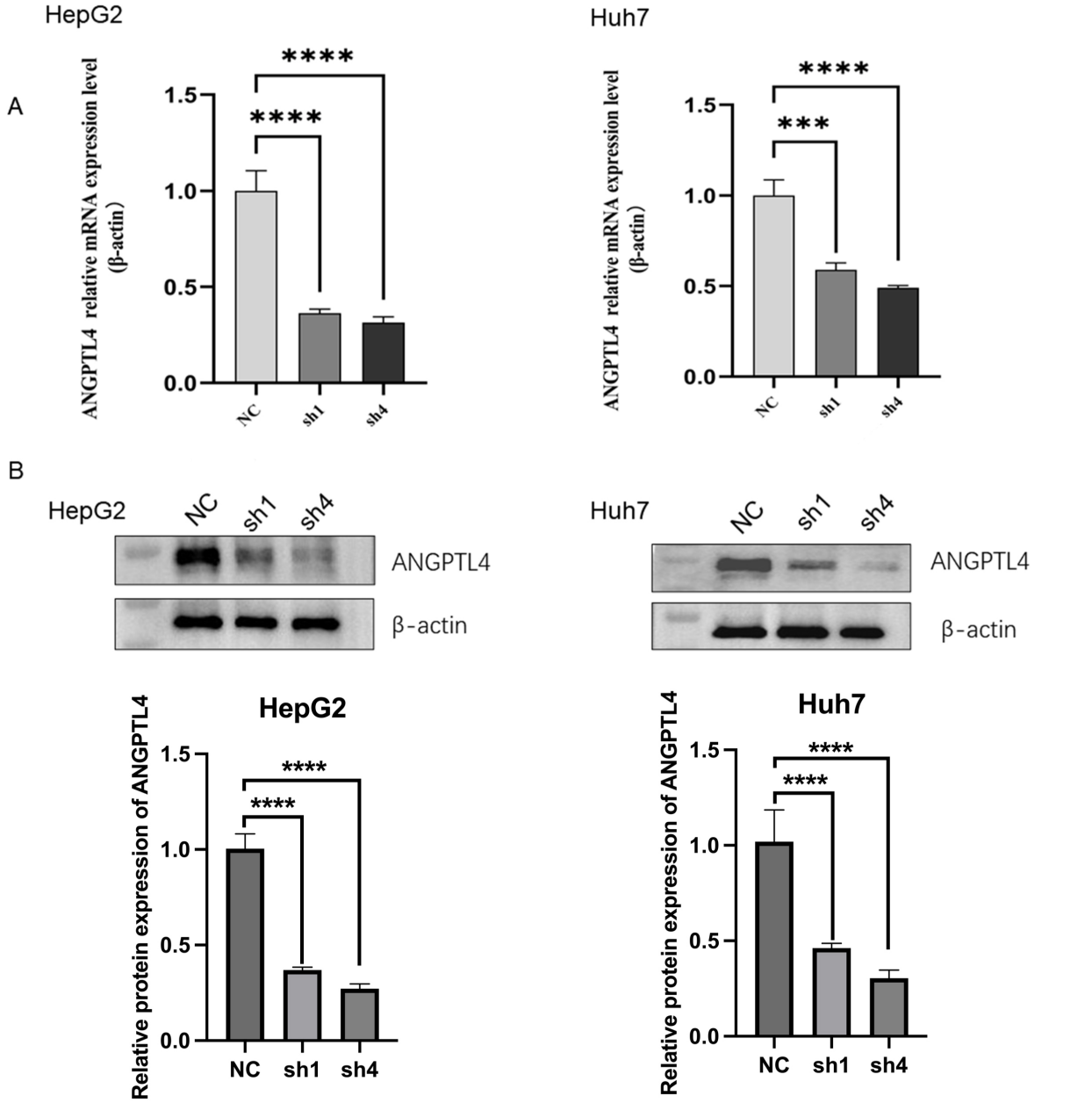

Fig. 2. Stable knockdown ANGPTL4 cell lines were constructed. (A) qRT-PCR on the relative mRNA level of ANGPTL4 in HepG2 and Huh7 cells transfected with two ANGPTL4 or control shRNAs (ANGPTL4-1, ANGPTL4-4, or ANGPTL4-NC, respectively). (B) Western blots of ANGPTL4 protein expression in the two cells lines transfected with two ANGPTL4 or control shRNAs. sh1, sh4 and NC stand for shRNA ANGPTL4-1, ANGPTL4-4, and ANGPTL4-NC, respectively. Data presented as the mean ± standard deviation. N = 3, ***$p < 0.001$, ****$p < 0.0001$. qRT-PCR, quantitative RT-PCR.

shown that abnormal tumor cell growth leads to poor vascularity and hypoxia in the tumor microenvironment. Hypoxia induces ANGPTL4 expression. Meanwhile, ectopic ANGPTL4 overexpression stimulates transendothelial migration of HCC cells *in vitro* and promotes intrahepatic and pulmonary metastasis *in vivo*. Knockdown of ANGPTL4 or the use of ANGPTL4-neutralizing antibodies reduced the metastatic potential of HCC cells *in vitro* [20]. Our results indicated that high expression of ANGPTL4 is essential for HCC proliferation.

In contrast to our findings, the ANGPTL4 mRNA level in tumor tissues of HCC patients was lower [21]. This may be due to the fact that the mRNA and protein expression levels reflect pretty different aspects of the condition, with the former indicating the expression of ANGPTL4 at local sites in the tumor, while the latter represents the overall expression of the tumor, the kidney or even the whole body. However, they also concluded that ANGPTL4 can be an important diagnostic and prognostic biomarker for patients with HCC and that overexpression of ANGPTL4 has

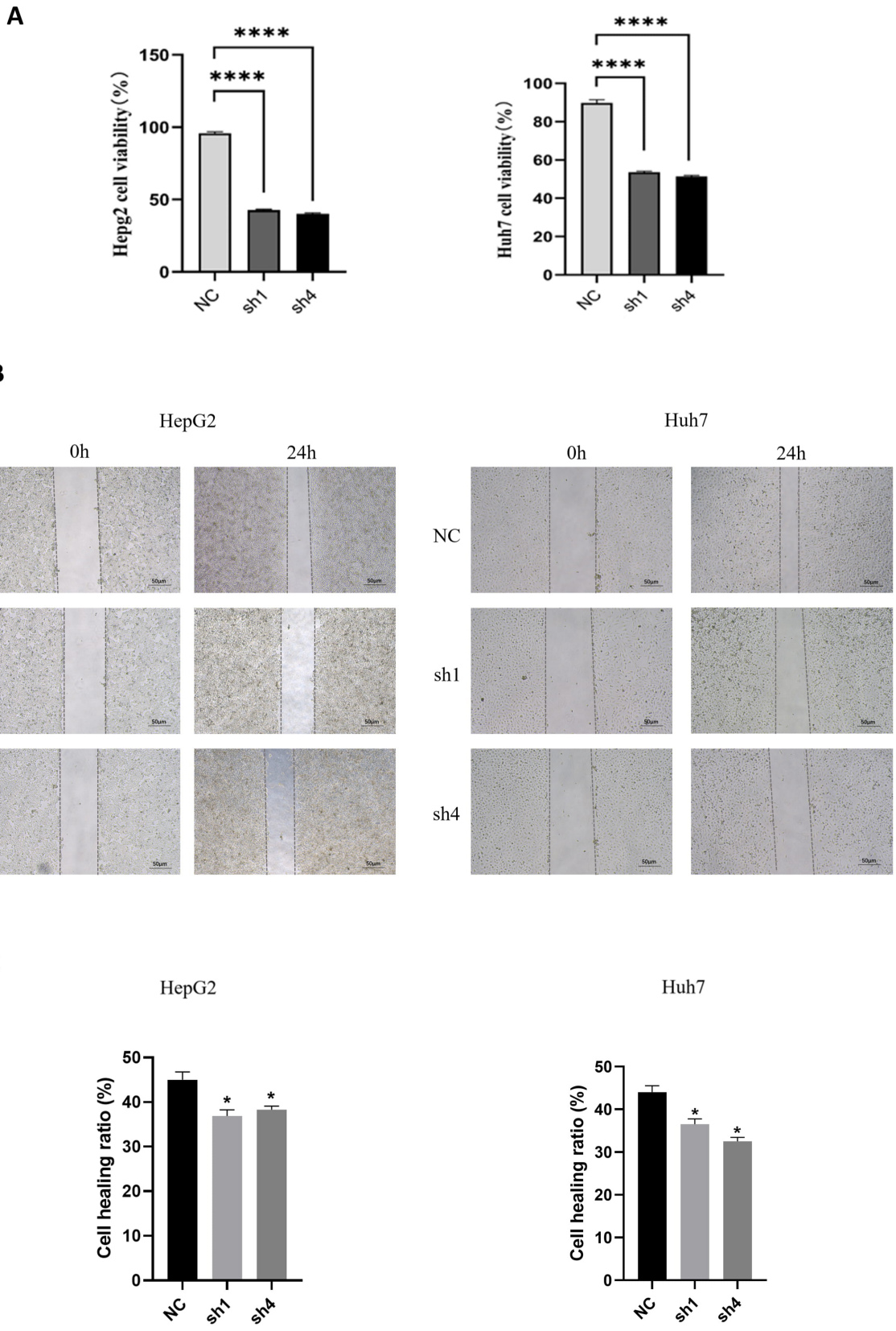

Fig. 3. Effect of knockdown of ANGPTL4 on cell viability and migration ability of HepG2 and Huh7 cells. (A) CCK-8 assay was used to detect the effect of different shRNA knockdowns of ANGPTL4 on cell viability in HepG2 and Huh7 cell lines. (B) Migration plots of each group of cells in the scratch assay. Magnifier 10×, scale bar = 50 µm. (C) Statistical graph of cell migration rate for each group. sh1, sh4 and NC stand for shRNA ANGPTL4-1, ANGPTL4-4, and ANGPTL4-NC, respectively. Data presented as the mean ± standard deviation. N = 3, *$p < 0.05$, ****$p < 0.0001$. CCK-8, Cell Count Kit-8.

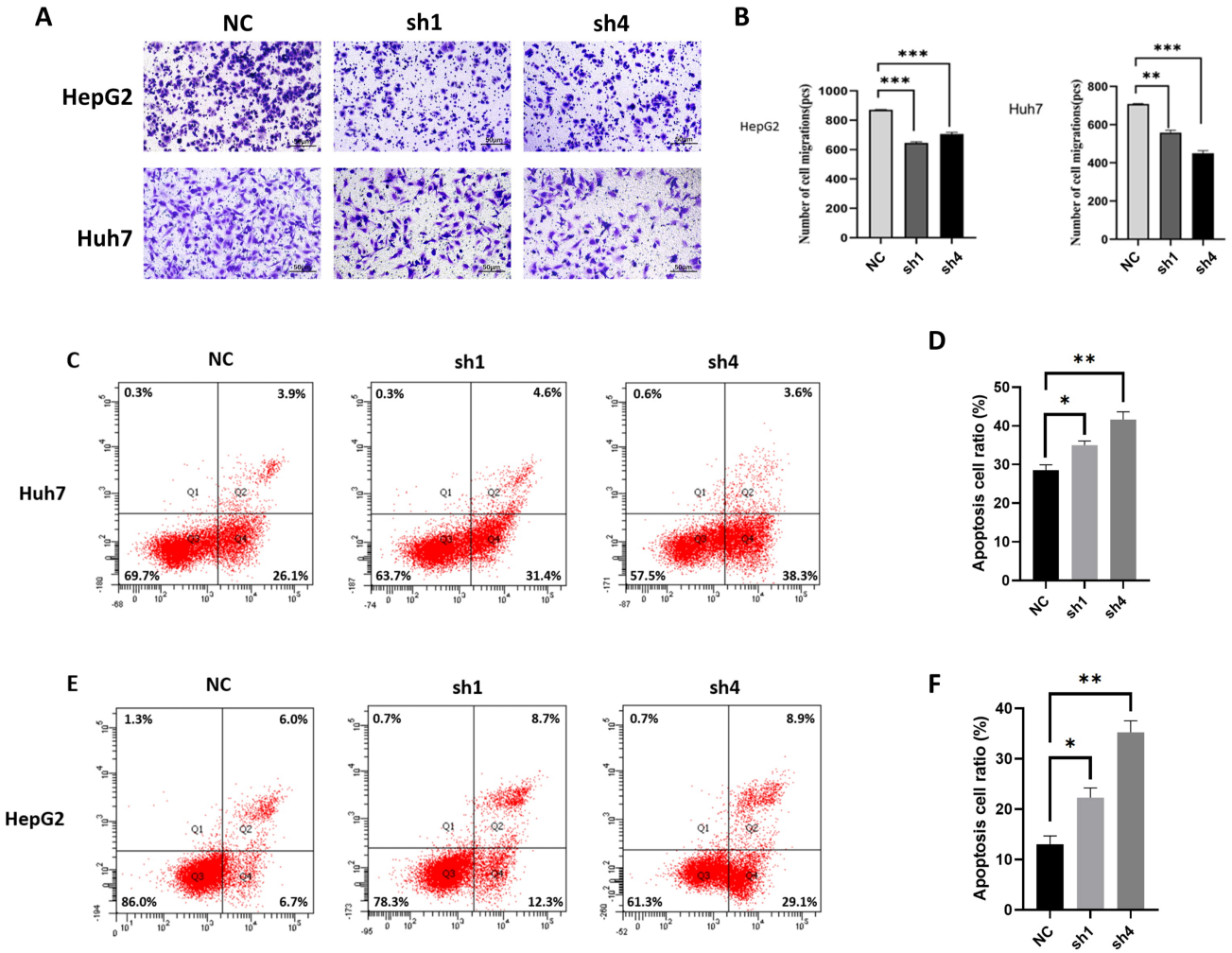

Fig. 4. Effect of knockdown of ANGPTL4 on invasion and apoptosis of HepG2 and Huh7 cells. Transwell cell invasion assay (A). Transwell cell invasion plots (B). Invasion counts of each cell group. The effect of different shRNA knockdown ANGPTL4 on apoptosis in HepG2 and Huh7 cell lines was detected by flow cytometry (C). Flow cytometry detection of apoptosis plots in Huh7 cells (D). Apoptosis counts of each group of cells in Huh7 cells (E). Flow cytometry detection of apoptosis plots in HepG2 cells (F). Apoptosis counts of each group of cells in HepG2 cells. sh1, sh4 and NC stand for shRNA ANGPTL4-1, ANGPTL4-4, and ANGPTL4-NC, respectively. Data presented as the mean ± standard deviation. N = 3, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

been shown to inhibit tumor growth by stimulating apoptosis [21]. ANGPTL4 disrupts endothelial cell-epithelial cell interactions, enhances vascular leakage, and promotes metastasis of cancer cells [22,23].

Conclusions

This study indicated that the ANGPTL4 gene is overexpressed during the development of hepatocellular carcinoma and that increased ANGPTL4 expression is significantly related to the HCC. In conclusion, ANGPTL4 may function in regulating the development of HCC, and ANGPTL4 can be used as a molecular marker for the clinical screening of HCC.

Availability of Data and Materials

Data will be available from the corresponding author upon requests.

Author Contributions

YB, GC and YY designed the research study. YB, GC, XS and MW performed the research. MW, YL and JG analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The protocol of this study was approved by the Ethics Review Committee of the Second Hospital of Harbin Medical University [Committee of the Second Hospital of Harbin Medical University KY-2022-208] in accordance with the Declaration of Helsinki, and informed consent was obtained from the patients themselves or their legal guardians.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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