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miR-409 down-regulates Jak-Stat pathway to inhibit progression of liver cancer

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Abstract. – OBJECTIVE: To elucidate the influence of microRNA-409 and the Jak-Stat pathway on the development of liver cancer.

PATIENTS AND METHODS: The quantitative Real-time polymerase chain reaction (qRT-PCR) method was used to detect the expression of microRNA-409 in hepatocarcinoma, paracancerous tissues and normal liver tissues, and the correlation between its expression and clinicopathological parameters of patients was analyzed, with the area under the microRNA-409 curve (AUC) being detected. The level of microRNA-409 in different liver cancer cells was detected by qPCR. Then it was overexpressed or knock-downed in the liver cancer cells by cell transfection technique. The cell apoptosis and viability after inhibition or overexpression of microRNA-409 were evaluated by propidium iodide (PI) staining and cell counting kit-8 (CCK-8) assay. Subsequently, Jak2 and Stat3 mRNA levels were detected by qPCR in hepatocarcinoma and paracancerous tissues, with their protein levels analyzed by Western blot after microRNA-409 was inhibited or up-regulated. At last, CCK-8 assay was performed to evaluate the effect of Jak2 on cell viability.

RESULTS: Compared with paracancerous and normal liver tissues, the level of microRNA-409 was remarkably reduced in hepatocarcinoma tissues and was negatively correlated with tumor stage, tumor size and overall survival time of patients with liver cancer. Meanwhile, microRNA-409 expression in hepatoma cell lines was also strikingly lower than that in normal liver cells. After overexpression of microRNA-409 in HHCC, cell viability significantly decreased while apoptosis increased, and opposite results were shown in HepG2 cells after miR409 was knock-downed. In liver cancer tissues, the levels of Jak2 and Stat3 were significantly higher than those in adjacent tissues. Additionally, up-regulating microRNA-409 reduced the level of Jak2 and Stat3 protein, while down-regulating it elevated them. In addition, Jak2 could reverse the inhibitory effect of microRNA-409 on the proliferation of hepatoma cells.

CONCLUSIONS: Highly-expressed microRNA-409 can down-regulate the Jak-Stat signaling pathway and inhibit cell proliferation to slow down the progression of liver cancer.

Key Words
MicroRNA-409, Jak-Stat signaling pathway, Liver cancer, Proliferation.

Introduction

Liver cancer is one of the most common malignant tumors in the world. The number of new cases of liver cancer has reached 782,500 per year, and this figure shows an upward trend[1,2]. Although the level of diagnosis and treatment of liver cancer has been increasing in recent years, the pathogenesis of liver cancer is very complicated, and the treatment effect is still not satisfactory. At present, surgical treatment is the main method for the treatment of liver cancer, but most patients with liver cancer have already been in the advanced stage at the time of diagnosis. They have lost the opportunity of surgical treatment and can only take radiotherapy, chemotherapy, transcatheter arterial chemoembolization or targeted therapy[3,4]. Unfortunately, liver cancer is not sensitive to radiotherapy and chemotherapy. Targeted drug therapy also has problems with unclear indications, and the treatment effect is very limited, which makes the prognosis of liver cancer poor and the status of recurrence not fundamentally changed. Therefore, looking for new therapeutic targets is urgent.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs with a length of about 17 to 25 bp. The expression of miRNAs is widely detected

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in plants and animals. In the human genome, 30% of genes are regulated by miRNAs[5]. MiRNAs can regulate the diversity of life processes, involving cell cycle, differentiation, development and metabolism[6,7], as well as human diseases such as diabetes mellitus, immune or neurological disorders, especially cancer[8,9]. MiRNAs can be involved in the development and progression of tumors as oncogenes or tumor suppressors. MiRNAs which negatively regulate the tumor suppressor genes are up-regulated in tumor development to silence their expression. On the contrary, miRNAs that inhibit the expression of cancer-promoting genes are down-regulated as tumor developing, knockout or mutation of these miRNAs may lead to overexpression of cancer-promoting genes[10,11]. Studies have found that miRNAs exhibit different functional characteristics in the regulation of tumor progression. Abnormal expression of some miRNAs is associated with tumorigenesis. For example, miR-122 has low expression in liver cancer, and up-regulation of its level can induce apoptosis and inhibit proliferation of HepG2 cells[12]. The level of miR-582 is down-regulated in hepatocarcinoma tissues, and its up-regulation can inhibit tumor cell proliferation, making the cell cycle arrested in G0/G1 phase[13]. The overexpression of miR-221 can enhance the growth, migration and invasion of liver cancer[14]. The miR-21 is an oncogene that is expressed most frequently and highly in liver cancer. It can promote metastasis and invasive ability of tumor cells after being up-regulated by various factors such as hypoxia and inflammation, and can also regulate kinase through the AKT signaling pathway to promote cell proliferation[15].

The JAK tyrosine kinase-signal transducers and activators of transcription (JAK-STAT) pathway, as a new and extremely fast signaling pathway, can deliver extracellular signals to the nucleus. This is achieved by the activation of the receptor-tyrosine kinase-signaling and transcriptional activator-target genes, ultimately eliciting biological effects. Under physiological conditions, the JAK-STAT signaling pathway plays a decisive role in cell growth, differentiation[16], tissue and organ formation, and immune defense barriers. At present, in the study of human solid tumors such as breast cancer, colon cancer, prostate cancer[17-19], the JAK-STAT signaling pathway is found constitutively activated and can participate in the occurrence, development, invasion and metastasis of tumors. Studies have shown that blocking JAK-STAT signaling can inhibit the proliferation of prostate cancer stem cells[20]. Phosphorylation proteome analysis revealed a key role for JAK-STAT signaling in the maintenance of breast cancer chemotherapy[21]. In addition, the growth of recurrent pancreatic cancer in mice can be inhibited by JAK-STAT pathway[22]. Similarly, the levels of STAT1, STAT3 and STAT5 in human hepatocarcinoma tissues were significantly higher than those in adjacent tissues. And STAT3 protein level was positively correlated with the grade and clinical stage of liver cancer; the higher the level of phosphorylated STAT3 (p-STAT3), the worse the prognosis[23].

In summary, miRNAs and the JAK-STAT signaling pathway are closely related to the incidence, development, metastasis, staging, prognosis, cell proliferation, cell differentiation and apoptosis of liver cancer. Therefore, further exploring the relationship between miRNAs and JAK-STAT signaling pathway should become a bright spot and a new treatment of liver cancer.

Patients and Methods

General Information of Subjects

Tissue samples were collected from 64 patients with liver cancer who underwent surgical resection during July 2014 to July 2017 of hospitalization in the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Another 28 normal liver tissues were derived from organ donors. All patients received no treatment before surgery. All cancer specimens were strictly verified by the pathologists of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. The paracancerous tissues were taken from 2 cm away from the edge of the tumor. Then they were put into tissue cryotubes and immediately stored in liquid nitrogen for RNA and protein extraction. The collection of tissue specimens was approved by the subjects and hospital Ethics Committee.

Cell Culture

Three liver cancer cell lines including HepG2, HHCC, HB611 and one normal immortalized liver cell L-O2 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cell L-O2 was cultured in a 1640 medium containing 10% FBS. HepG2, HHCC and HB611 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) high glucose medium containing 10% fetal bo-

vine serum (FBS). The cells were observed daily, and the medium was replaced every 1-2 days. Cells were passaged when they reached about 80% of confluence.

Transfection Experiment

The appropriate number of cells was seeded in culture plates one day before the transfection, and the cell density at the time of transfection was controlled to be about 50%. A mixture of microRNA-409 mimic/inhibitor and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was prepared and added to a culture plate containing cells, shaken slowly, and placed in a 37°C, 5% CO_2 incubator. After 4-6 h, the medium containing the transfection mixture was aspirated, added to fresh medium, and subjected to subsequent experiments. The transfected cells were divided into three groups. HepG2 cells with high expression of microRNA-409 were transfected with microRNA-409 inhibitor or simultaneously transfected with Jak2 overexpression plasmid, and HHCC cells with low expression of microRNA-409 were transfected with microRNA-409 mimic or simultaneously Jak2 small interference sequence was transfected as well as the negative control group.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA of tissues or cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed to obtain complementary Deoxyribose Nucleic Acid (cDNA) using reverse transcription kit. QPCR was performed with SYBR Green nucleic acid fluorescent dyes according to the system, and the relative expression levels of microRNA-409, Stat3 and JAK2 were calculated by the $2^{-\Delta\Delta CT}$ method. The primers were as follows: GAPDH (F): 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; GAPDH (R): 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; miR-409 (F): 5'-GGGGTTGGAATCGTTGGA-3'; miR-409 (R): 5'-GGCCTCCCTGGTGGAATT-3'; Stat3 (F): 5'-GGAGGAGGCATTCGGAAAG-3'; Stat3 (R): 5'-TCGTTGGTGTCACACGAT-3'; Jak2 (F): 5'-AAGCAAGCAAACCAAGAGGG-3'; Jak2 (R): 5'-TCACTAAGTTTGATGAAAGGAGGATT-3';

Cell Counting Kit-8 (CCK-8) Test

The cells in log phase growth phase were seeded into a 96-well culture plate with 4000 cells per well with 100 μL of culture medium. The cells were cultured at 37°C in a 5% CO_2 incubator and tested at 4 time points (24, 48, 72, 96 h). 10 μL of CKK-8 solution (Dojindo, Kumamoto, Japan) was added to each well. After 4 hours, the absorbance optical density (OD) at 450 nm was measured using a microplate reader, and the experiment was repeated 3 times. Cell viability % = (OD value of experimental group – OD value of control group) / (OD value of control group – OD value of blank group) × 100%.

Western Blot Assay

The tissues were lysed with radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) for extracting total proteins. They were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The PVDF membrane was blocked with 5% skim milk powder for 2 h, then primary antibodies (Jak2, Stat3 and GAPDH) were respectively added and incubated on a 4°C shaker overnight. On the next day, the secondary antibody was used to incubate for 2 h at room temperature. The PVDF membrane was placed in the Enhanced chemiluminescence reagent (ECL) for 1-3 min (Thermo Fisher Scientific, Waltham, MA, USA), and the proteins were exposed using a chemiluminescent gel imaging system. The gray value of these strips was finally scanned.

Detection of Cell Apoptosis

Cells were collected and resuspended by adding 180 μL of annexin V-fluorescein isothiocyanate (Annexin V-FITC) buffer. 5 μL of Annexin V-FITC was then aspirated for incubation for 10min at room temperature in the dark. Subsequently, 10 μL of propidium iodide (PI) was added at room temperature and mixed well, and then incubated for 30 min in the dark. The cells were then washed 3 times with phosphate-buffered saline (PBS) and the cell apoptosis was analyzed by flow cytometry.

Statistics Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Paired *t*-test was used for comparison between the two groups. Chi-square test was used for classification data. $p<0.05$ was considered statistically significant.

Results

MicroRNA-409 had a Low Expression in Liver Cancer Tissue

To explore the role of microRNA-409 in the development of liver cancer, qRT-PCR was first performed to analyze the microRNA-409 level in hepatocarcinoma, paracancerous and normal liver tissues. The results showed that in 64 cases of HCC, microRNA-409 level was significantly lower in cancer tissues than that in corresponding adjacent tissues or normal liver tissues (Figure 1A, 1B). The clinical pathological parameters (including age, gender, tumor size, TNM stage, tumor location, lymph node metastasis) of 64 subjects were collected, as shown in Table I, and the level of microRNA-409 was found negatively correlated with TMN stage and tumor size. In addition, the overall survival time of subjects with highly-expressed microRNA-409 was higher than those with lowly-expressed one (Figure 1C). Therefore, its expression could be used to diagnose the benign and malignant liver cancer (Figure 1D). The area under the curve (AUC) of microRNA-409 was 0.8816.

The Inhibitory Effect of microRNA-409 on the Apoptosis and Proliferation of Hepatoma Cells

To further explore the influence of microRNA-409 on hepatoma cells, we conducted qRT-PCR assay to evaluate microRNA-409 level in three common hepatoma cell lines including HepG2, HHCC, HB611 and one immortalized liver cell line L-O2. It was shown that the levels of microRNA-409 in HHCC and HB611 were significantly lower than those in normal liver cells (Figure 2A). HepG2 cells with the highest expression of microRNA-409 and HHCC cells with the lowest were subsequently selected for subsequent experiments. The stable transfected cell line with overexpression and low expression of microRNA-409 was established by lentivirus carrying microRNA-409 mimic and inhibitor, and negative control was established with lentivirus carrying blank control. qRT-PCR verified the validity of the establishment of the above cell lines. The microRNA-409 level was remarkably up-regulated when microRNA-409 mimic was transfected in HHCC, and was strikingly reduced

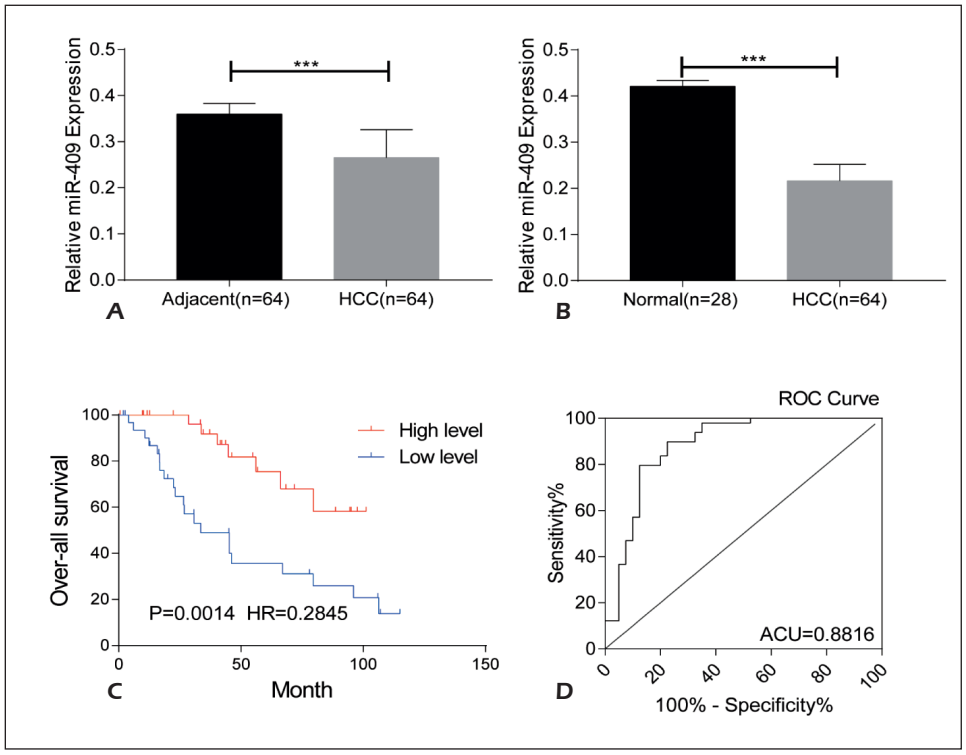

Figure 1. MiR-409 was lowly expressed in liver cancer. **A**, In 64 pairs of liver cancer tissues and adjacent tissues, miR-409 was down-regulated in liver cancer tissues. **B**, MiR-409 was less expressed in liver cancer tissues than normal liver tissues. **C**, The overall survival time of patients in miR-409 high expression group was longer than those in miR-409 low expression group. **D**, MiR-409 ROC curve in liver cancer tissues and adjacent tissues was shown (AUC = 0.8816).

Table I. Relations of miR-409 expression and the clinical pathological parameters in patients with hepatocarcinoma.

Clinicopathologic features	Number (n=64)	miR-409 expression		*p*-value
		Low (n=32)	High (n=32)	
Age (years)				0.2807
≤60	20	8	12	
>60	44	24	20	
Gender				0.3087
Male	26	11	15	
Female	38	21	17	
Tumor size				0.0238*
>5 cm	29	19	10	
≤5 cm	35	13	22	
TNM stage				0.0190*
III-IV	23	16	7	
I-II	41	16	25	
Histological classification				0.4530
Low grade	31	17	14	
Medium and high grade	33	15	18	
Lymph node metastasis				0.7945
Absent	23	11	12	
Present	41	21	20	

*$p<0.05$

after miR-210 inhibitor was transfected in HepG2 cells (Figure 2C). Apoptosis assay indicated an increased apoptosis in HHCC cells after microRNA-409 was up-regulated, and a decreased apoptosis in HepG2 cells following the inhibition of microRNA-409 expression (Figure 2B). The cell viability measured by CCK-8 test showed an opposite trend in HHCC cells and HepG2 cells (Figure 2D).

MicroRNA-409 Acted on the Jak-Stat Pathway to Inhibit the Progression of Liver Cancer

To further confirm the role of the Jak-Stat signaling pathway in the development of hepatocarcinoma, Jak2 and Stat3 levels were also detected by qRT-PCR in liver cancer tissues and adjacent tissues. It was demonstrated that Jak2 and Stat3 levels were markedly higher in the 64 cases of HCC than in the corresponding adjacent tissues (Figure 3A, 3B). Next, the correlation between Jak-Stat signaling pathway and microRNA-409 analyzed by Western blot revealed that the expression of Jak2 and Stat3 protein decreased after overexpression of microRNA-409, while opposite result was observed after the inhibition of microRNA-409 (Figure 3C). CCK-8 experiments revealed that Jak2 reversed the inhibitory role of microRNA-409 in the proliferation of hepatoma cells (Figure 3D). The above results indicated that high expression of microRNA-409 can down-regulate the Jak-Stat pathway and repress the development of liver cancer.

Discussion

Controlling liver cancer requires continuous improvement of high-quality screening, diagnosis and treatment methods. Although great progress has been made, it has not achieved the desired results yet. The search for new therapeutic targets for liver cancer has become a hot topic worldwide. Related researches have demonstrated that miRNAs are widely involved in the development of tumors[24,25], indicating that they can be used to diagnose and treat various tumors. MiRNAs are a class of non-coding small RNAs that inhibit translation or promote degradation of target mRNAs by combining with their complementary sequences. One kind of miRNA may affect the expression of hundreds of target genes. Thus, miRNAs have become a key factor in many diseases including tumors. At present, reports on liver cancer-related miRNAs are increasing. For example, studies have found that miR-364 promotes the proliferation, migration and invasion of liver cancer cells[26]. MicroRNA-221 promotes malignant progression of liver cancer by regulating the expression of HDAC6[27]. MiR-500a promotes

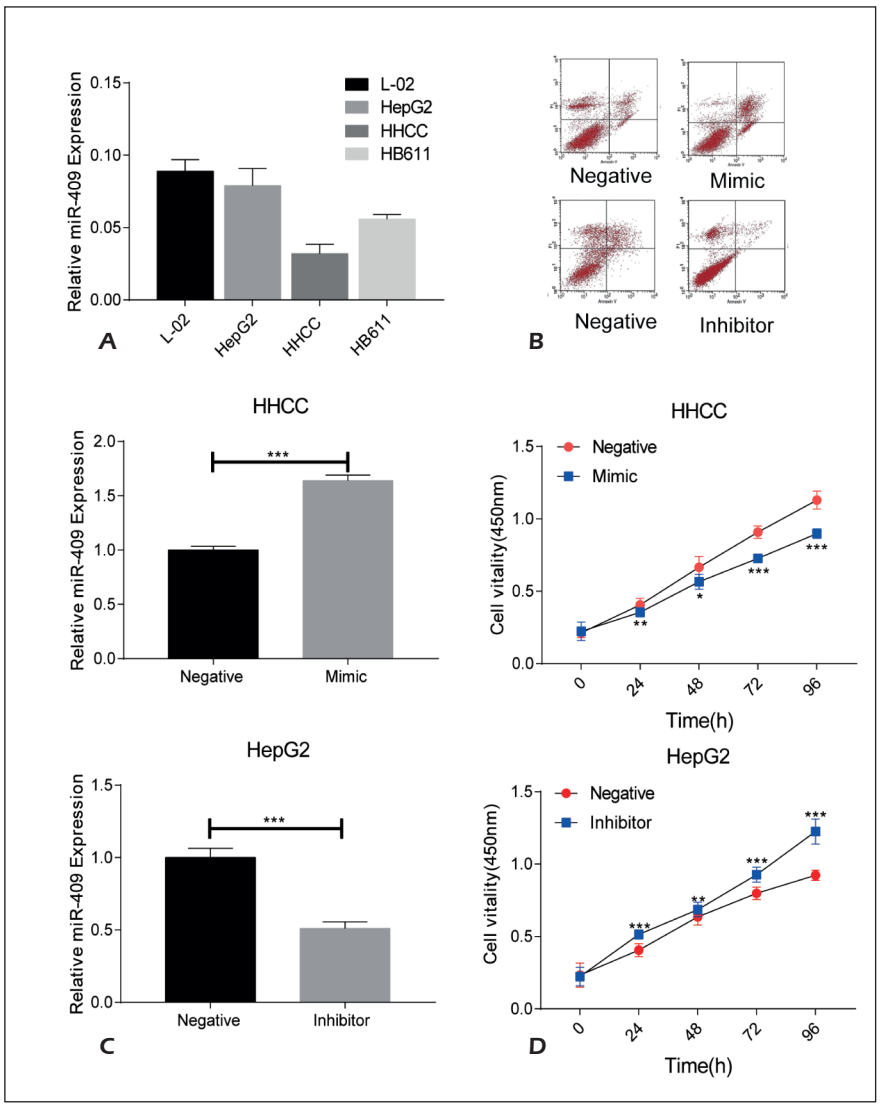

Figure 2. MiR-409 inhibited hepatoma cell proliferation and promoted cell apoptosis. **A**, Expression of miR-409 in normal liver cells and liver cancer cell lines (L-02, HepG2, HB611, HHCC) was detected. **B**, Overexpression of miR-409 increased apoptosis of HHCC cells, while inhibiting miR-409 expression decreased apoptosis of HepG2 cells. **C**, MiR-409 mimic and inhibitor were transfected in HepG2, HHCC cells. **D**, Overexpression of miR-409 reduced HHCC cell viability, while the inhibition of miR-409 expression enhanced HepG2 cell viability.

cancer progression by targeting BH3-interacting death agonist (BID) in hepatocellular carcinoma[28]. And miR-125b can directly act on the oncogene LIN28B2 to inhibit the proliferation and metastasis of human hepatoma cells[29]. Therefore, the discovery of miRNAs as a therapeutic target for liver cancer has received increasing attention, and related research is becoming more developed.

MicroRNA-409 is located on chromosome 14q32.31 and has been reported that stromal fibroblast-derived microRNA-409 can promote EMT and prostate tumorigenesis[30]. Subsequent reports indicate that this miRNA plays a role in inhibiting cancer progression in a variety of tumors. Studies have shown that microRNA-409 represses tumor cell invasion and metastasis in gastric cancer[31] and suppresses the proliferation of cervical cancer cells by regulating AKT[32]. In addition, it can target E74-like factor 2 in osteosarcoma to regulate cell proliferation and tumor growth[33]. Moreover, microRNA-409-3p can promote the proliferation and apoptosis of lung cancer cells through the target gene c-Met[34].

The Jak-Stat signaling pathway is an extremely rapid signal transduction pathway from extracellular to the nucleus. Current research believes the Jak-Stat signal transduction pathway is a key factor in the malignant tumor development, and is also involved in the proliferation, differentiation, development and metastasis of tumor cells. Studies have demonstrated that the Jak2/Stat3 signaling pathway can up-regulate the levels of VEGF and bFGF factor in non-small cell carcinomas and mediates tumor angiogenesis[35]. Moreover, the activation of the Jak2/Stat3 signaling pathway by bone porin is able to promote the

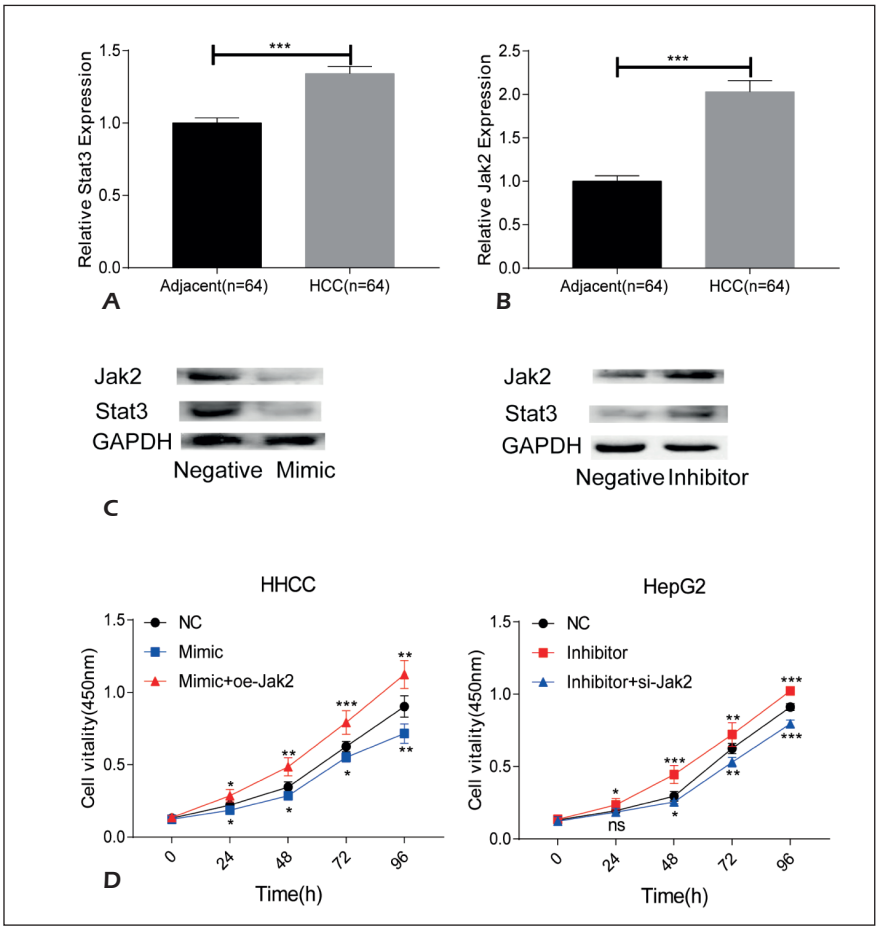

Figure 3. MiR-409 inhibited the Jak-Stat pathway to reppress progression of liver cancer. **A** and **B**, Expression of JAK2 and Stat3 was increased in 64 pairs of liver cancer tissues and adjacent tissues. **C**, After overexpression of miR-409, JAK2 and Stat3 protein level decreased, while the opposite result was observed after the inhibition of miR-409 expression. **D**, Jak2 can reverse the inhibitory effect of miR-409 on the proliferation of hepatoma cells.

growth of human breast cancer cells[36]. Simvastatin can inhibit the growth and metastasis of renal cancer cells *via* the AKT/mTOR, ERK and Jak2/Stat3 signaling pathways[37]. Fortunately, miRNAs are found to target the Jak2/Stat3 signaling pathway and thus regulate cancer progression. For instance, miR-375 is able to slow down the progression of gastric cancer caused by Helicobacter pylori by blocking the Jak2/Stat3 signaling pathway[38]. And miR-204 can target JAK2 to induce cell apoptosis of breast cancer[39].

In our study, we found that microRNA-409 was down-regulated in hepatocellular carcinoma cell lines, and its level in HCC was significantly lower than that in adjacent tissues and normal liver tissues. These results suggested that down-regulation of microRNA-409 may be involved in liver cancer progression. In addition, we applied cell transfection technology to knockdown or overexpress microRNA-409 in hepatocellular carcinoma cells. It was found that highly-expressed microRNA-409 reduced cell viability but elevated cell apoptosis, while opposite result was observed when microRNA-409 was down-regulated, proving that microRNA-409 could indeed inhibit the viability of liver cancer cells and promote their apoptosis. In addition, the levels of Jak2 and Stat3 in liver cancer tissues were also found significantly higher than those in adjacent tissues. Moreover, the protein levels of Jak2 and Stat3 were reduced when microRNA-409 was up-regulated and were enhanced when microRNA-409 was down-regulated.

Conclusions

We showed that the microRNA-409 can directly target the Jak-Stat3 pathway so as to regulate the development of liver cancer.

Conflict of Interests

The authors declare that they have no conflict of interest.

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