European Review for Medical and Pharmacological Sciences 2021; 25: 710-721

Correlation between inflammatory cytokines and liver cancer stem cell markers in DEN-induced liver cancer rats

Z.-H. LIU[1], M.-J. BAI[2], J.-Q. LI[3], S.-H. YAN[4], X.-W. YE[5], J.-G. BU[3], C.-Y. CHEN[6]

[1]Department of Oncology, Medical School of Chinese PLA, Chinese PLA General Hospital, Beijing, China
[2]Department of Vascular Interventional Radiology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, P.R. China
[3]Department of Radiotherapy, Zhujiang Hospital, Southern Medical University, Guangzhou, P.R. China
[4]Department of Thoracic Oncology, Qinhuangdao Fourth Hospital, Qinhuangdao, P.R. China
[5]Department of Oncology, First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, P.R. China
[6]Department of Intervention, Xiangya Hospital, Central South University, Changsha, China

Zihao Liu, Mingjun Bai and Jiqiang Li contributed equally to this study

Abstract. – OBJECTIVE: To study the association of inflammatory factors and hepatocarcinoma stem cells of induced liver cancer rats.

MATERIALS AND METHODS: 30 SD male healthy rats were selected. 10 rats were given water as normal control group. 10 rats only were implemented laparotomy as sham operation group. The remaining 10 rats were the liver cancer model group and treated with diethylnitrosamine (DEN) to induce liver cancer. Real-time quantitative PCR was used to detect the related inflammatory factors in HCC tissues, including interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β1 (TGF-β), human interleukin-1α (IL-1α), human interleukin 1β (IL-1β) and levels of hepatocarcinoma stem cells indicators CD90, CD133, Alpha-fetoprotein (AFP). Correlation analysis was used to analyze the correlation between inflammatory factors and hepatocarcinoma stem cells markers CD90 and CD133.

RESULTS: The expression levels of IL-6, MCP-1 and TGF-β of HCC tissues in liver cancer model group were significantly higher than those in the control group and the sham operation group. The expression levels of CD90 and CD133 of tissues in the liver cancer model group were significantly higher than those in the control group and the sham operation group. The differences were statistically significant ($p<0.001$). By inhibiting related inflammatory factors, the growth, migration and invasion of liver cancer cells were significantly inhibited, and apoptosis was promoted. Correlation analysis results showed that the expression changes of IL-6, MCP-1 and TGF-β were significantly positively correlated with CD90 up-regulation ($p<0.05$), while the expression changes of IL-6, MCP-1 and TGF-β were significantly positively correlated with CD133 up-regulation ($p<0.05$).

CONCLUSIONS: The inflammatory factors IL-6, MCP-1 and TGF-β are closely related to hepatocarcinoma stem cells, which plays an important role in promoting the occurrence and deterioration of liver cancer.

Key Words:
Induced liver cancer, IL-6, MCP-1, TGF-β, Cancer stem cells CD133 and CD90.

Introduction

Liver cancer is a common invasive cancer with high recurrence rate and heterogeneity[1], which is generally more common in males[2]. Liver cancer is also the second most common cause of cancer deaths worldwide. The ratio of mortality to incidence of liver cancer is 0.95, which indicates a poor prognosis[3]. Generally, liver cancer lesions can be completely removed when detected in the early stage. However, because the early symptoms of liver cancer are not evident, the disease is already in the deterioration stage when it is discovered.

Cancer stem cells have been defined as a small part of cancer cells *in vivo* of tumors that exhibit

Corresponding Authors: Junguo Bu, MD; e-mail: 755591296@qq.com
Changyong Chen, BSA; e-mail: 13874991059@163.com

self-renewal and differentiation. Differentiation of cancer stem cells may contribute to tumor initiation, metastasis, recurrence, and drug resistance[4,5]. Cancer stem cells have been proposed as the initial cells of cancer and have been verified to be important causes of chemotherapy drug resistance and cancer recurrence[6].

Inflammation has been recognized as sign of cancer and is known to play an important role in the deterioration and progression of most tumors, even those that have no visible signs of inflammation and infection[7]. Kupffer cells are macrophages that are resident in the liver and play a key role in maintaining liver function. The special functions and metabolism of Kupffer cells suggest that they are attractive targets for the treatment of hepatic inflammation and related diseases, including cancer and infectious diseases[8]. Kupffer cells release Interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β) and other inflammatory factors to involve response when Kupffer cells participate in the immune response[9-11]; besides, inflammation plays a decisive role in tumor deterioration[12,13]. Inflammatory factors participate in the self-renewal of tumor stem cells and transformation of epithelial-mesenchymal, which promotes the deterioration of tumors[14,15].

The development of medical technology brings new treatments for liver cancer, but the prognosis is still not satisfactory. If the relationship between inflammatory factors and hepatocarcinoma stem cells can be found, it may provide new directions for treatment of liver cancer in clinic. Therefore, this study aimed to understand the changes of inflammatory factors and hepatocytes of rats by induced liver cancer.

Materials and Methods

Materials and Reagents

SD male rats were purchased from Wuhan Yun Kuan Diagnostic Reagent Research Institute Co., Ltd (Wuhan, Hubei, China), and Human hepatoma cell line Hep G2 was from Wuhan Yipu Biotechnology Co., Ltd (Wuhan, Hubei, China). Phosphate buffered saline (PBS) was purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd (Shanghai, China). PrimeScript RT reagent Kit reverse transcription kit was purchased from Beijing winter song Boye Biotechnology Co. Ltd (Beijing, China). Diethylnitrosamine (DEN, 0.95 g/ml) was purchased from Shanghai YuanMu Biological Technology Co. Ltd (Shanghai, China). TRIzol reagent was purchased from Changsha Daerfeng Biotechnology Co., Ltd (Changsha, Hunan, China). SYBR Green kit was purchased from Beijing Jiehui Bogao Biotechnology Ltd (Beijing, China). Real-time quantitative PCR instrument was purchased from Beijing Jiamei Qianyuan Biotechnology Co., Ltd (Beijing, China). SimpliNano micro spectrophotometer was purchased from Jiangsu Bomeida Life Science Co., Ltd (Suzhou, Jiangsu, China). Apoptosis kit (Shanghai Yanqi Biological Technology Co., Ltd (Shanghai, China).), T10% fetal bovine serum (FBS; Shanghai So-Fe Biomedical Co., Ltd., Shanghai, China), Dilbecco's Modified Eagle's Medium (DMEM) medium (Qingdao Jisskang Biotechnology Co., Ltd. (Qingdao, Shandong, China)), Transfection reagent Lipofectamine TM2000 (US Everbright Inc.,Suzhou, Jiangsu, China), CCK8 kit (Shanghai Jingkang Biological Engineering Co., Ltd., Shanghai, China), UV spectrophotometer (Beijing Jiayuan Industrial Technology Co., Ltd., Beijing, China), BD flow cytometer (Shanghai Binzhi Biological Technology Co., Ltd., Shanghai, China), transwell chamber (Shanghai SunBio Biomedical technology Co., Ltd., Shanghai, China), Reverse transcriptase (Shanghai Shifeng Biological Technology Co., Ltd., Shanghai, China), Microplate reader (Beijing Image Trading Co., Ltd., Beijing, China), β-catenin, cyclin D1, c-myc primary antibodies (K000226M, bs-0623R-1, bs-4963R-1, Shanghai Zhenyu Biological Technology Co., Ltd., Shanghai, China), HRP-labeled goat anti-mouse secondary antibody (LD-BJ-101891, Shandong Lvdu Bio-Sciences & Technology Co., Ltd, Binzhou, Shandong, China). Various primers were synthesized by Zhongmei Taihe Biotechnology co., LTD. (Beijing, China).

Methods

Establishing Rat Model of Induced Liver Cancer

Since DEN is highly toxic to humans and animals, even low-dose injections or oral administration can cause severe liver damage. Its tumorigenic cycle is short, and its pathological process follows the general development of human liver cancer[16], so rat model of liver cancer was established using DEN. There were 30 SD male healthy rats, 2 to 3 months, with a weight of 170-250 g. The rats were in good health with

no abnormalities. Ten rats were randomly included in normal control group. The normal control group was given water. Ten rats were divided into sham operation group and given generally water. The remaining 10 rats were included in liver cancer model group. Rats in the liver cancer model group were given water with a mixed concentration of 0.01% DEN solution for 6 consecutive days, and then given water for one day until the formation of liver cancer by inducing cancer at the 24th week. The rats were decapitated without anesthesia, and then the liver was removed by laparotomy. If the liver has scattered gray-white nodules, scattered blood spots on the surface, and the liver is darker, it is a successful model. This liver was refrigerated at 80°C for later use. The investigation was approved by the Hospital Animal Ethics Committee. The operation process was strictly in accordance with the guidelines for surgical care and use of experimental animals (People's Health Publishing, 2008 revision, ISBN: 9787117105071).

Detection of Inflammatory Factors in HCC Tissues and Expression Levels in Hepatocarcinoma Stem Cells Marker By Quantitative Real Time-PCR

Total RNA in HCC tissues was extracted by TRIzol strictly according to the instruction. Detect the concentration and purity of total RNA by UV spectrophotometer, take RNA with OD260/OD280 ratio between 1.8 and 2.0, and perform reverse transcription of total RNA using reverse transcription-fluorescence quantification kit according to the instructions. The cRNA was synthesized using reverse transcriptase and oligonucleotides according to the operating instruction. Transcription reaction system (20 μL) was 4 μL of buffer, 2 μL of reverse transcriptase, 2 μL of total RNA and 12 μL of de-RNase-Free water. Reaction conditions were water bath at 42°C for 1 hour and water bath at 95°C for 5 minutes. Amplification and quantification were performed using a PCR instrument with GAPDH as internal reference. According to the operating instructions, use relevant specific primers and use SYBR Green kit to quantitatively detect the expression levels of related inflammatory factors and liver cancer stem cell indicators released by Kupffer cells in real-time quantitative PCR. The PCR reaction system (20 μL) was 0.4 μL of the upstream primer, 0.4 μL of the downstream primer and 0.5 μL of the miR. The rest was filled with ddH_2O. Reaction conditions: pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s for a total of 40 cycles. 3 multiple pores were set up for each experiment and the experiment was repeated 3 times. The results were analyzed using the relative quantitative method. Relative expression was calculated using $2^{-\Delta\Delta CT}$. The above operations were carried out in strict accordance with the instructions. The primer sequences were reported in Table I.

The Detection of Inflammatory Factors and Relative Protein Expression Levels of Liver Cancer Stem Cell Markers by WB

The lysed cells were collected and transferred to a centrifuge tube, centrifuged at 12000 × g for 10 min at 4°C, and the supernatant was collected as a protein sample. The protein concentration was measured by bicinchoninic acid (BCA) method, and the protein sample was diluted by adding Lysis buffer to prepare 20 mg/ml protein. 8.00% separation gel and 5.00% laminated gel were prepared before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, then transfer to PVDF membrane. Add β-catenin, cyclin D1, c-myc (1:1000) primary antibodies, internal reference β-actin (1:3000), and block overnight at 4°C. Add horseradish peroxidase (HRP)-labeled goat anti-mouse secondary

Table I. Primers sequence.

Genes	Upstream primers	Downstream primers
IL-6	5'-TAGTCCTTCCTACCCCAACTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'
MCP-1	5'-ATGCAGGTCTCTGTCACGCTTCTGGGC-3'	5'-CTAGTTCTCTGTCATACTGGTCAC-3'
TGF-β	5'-GCTAATGGTGGACCGCAACAAC-3'	5'-CACTGCTTCCCGAATGTCTGAC-3'
Human Interleukin-1α (IL-1α)	5'-AGACCATCCAACCCAGATCA-3'	5'-TGATGAACTCCTGCTTGACG-3'
Human Interleukin-1β (IL-1β)	5'-GACCTGTTCTTTGAGGCTGACA-3'	5'-CTCATCTGGACAGCCCAAGTC-3'
CD90	5'-GCTGGATGGGCAAGTTGGT-3'	5'-TGACAGCCTGCCTGGTGAA-3'
CD133	5'-CCAGCGGCAGAAGCAGAACGA-3'	5'-GTCAGGAGAGCCCGCAAGTCT-3'
Alpha-fetoprotein (AFP)	5'-GTCCCTCCACCATTCTCTGA-3'	5'-GGTCTTTGCAGCACTTCTCC-3'
GAPDH	5'-TGCCACTCAGAAGACTGTGG-3'	5'-TTCAGCTCTGGGATGACCTT-3'

antibody (1:5000), incubate for 1 h at 37°C, rinse 3 times with Tris-Buffered Saline and Tween-20 (TBST) for 5 min each. Then, develop in a dark room, absorb excess liquid from the membrane. Enhanced chemiluminescence (ECL) was used to develop. The protein bands were scanned, and the gray values were analyzed using Quantity One software (Molecular Devices Corp, The Bay Area, CA, USA).

Cell Culture and Transfection

Transfect liver cancer cell line into DMEM medium containing 10% fetal bovine tissue and penicillin-streptomycin mixed solution. The growth conditions should be 37°C and 5% CO_2 in a cell incubator. Cells were seeded into a 6-well plate to achieve a cell density of 60% to 70%. The cells were separated into 4 groups: IL-6 inhibition group (IL-6 siRNA), MCP-1 inhibition group (MCP-1 siRNA), TGF-β inhibition group (TGF-β siRNA), and Control group (no-load plasmid). All siRNA plasmid vectors were purchased from Shanghai Yingbio Technology Co., Ltd. (Shanghai, China). Each group of inflammatory factor inhibitors was transfected into liver cancer cells using Lipofectamine™ 2000. IL-6, MCP-1, TGF-β inhibitors were mixed with 200 μl of FBS-free medium at room temperature for 15 minutes. The mixed lipo2000 was then incubated with IL-6, MCP-1, TGF-β inhibitors for 30 minutes at room temperature. After washing with PBS, the cells were treated with a mixture kept in FBS-free medium and incubated for 6 hours. After changing the medium, the cells were further cultured for 48 hours before being collected.

Detection of Cell Proliferation

The transfected Hep G2 cells were made into suspensions, 100 μl/well of cell suspension was seeded into 96-well plates, and three replicates were set in each well. Cell proliferation was detected at 24 h, 48 h, 72 h, and 96 h. Add 20 μL Cell Counting Kit reagent (CCK-8) to each well 2 h before the end of culture, place in a 37°C cell incubator, with 5% CO_2, and measure the absorbance at 490 nm using a fully automatic microplate reader after 2 h. The experiment was repeated three times.

Detection of Cell Migration and Invasion

Trypsinize cells first, then resuspend cells in serum-free medium. Take 200 μL of the resuspension, perform the migration assay, and place approximately 5×10^4 cells in the upper chamber. Take 200 μL of the resuspension for invasion experiments, containing approximately 5×10^4 cells, and add 1 m of FBS-containing medium to the lower chamber of the 6-well plate. After 24 hours of routine incubation, the cells in the upper chamber of transwell were wiped off with a cotton swab. Cells that migrated to the lower chamber were stained with 4% paraformaldehyde and stained with 0.1% crystal violet. After the transwell microscope. The experiment was repeated three times.

Apoptosis Detection

The apoptosis detection kit was used to detect the apoptosis of the cells in accordance with the instructions. BD flow cytometry was used to detect cells that had been transfected for 48 h and stained with AnnexinV, propidium iodide (PI) in the 6-well plate. The experiment was repeated 3 times.

Statistical Analysis

Statistical analysis was performed for data using SPSS 21.0 (Asia Analytics Formerly SPSS China). The measurement data were expressed as mean number ± standard deviation (x ± SD). The measurement data were compared by *t*-test between two groups. One-way ANOVA followed by LSD-*t* test was used for multiple comparisons. Pearson correlation analysis was used to analyze the correlation between inflammatory factors and liver cancer stem cell markers. The difference was statistically significant with $p<0.05$.

Results

Expression Levels of Inflammatory Factors in Each Group Tissues

The expression levels of related inflammatory factors in rats HCC tissues were detected by quantitative PCR. The results showed that IL-6 expression in the control group, sham operation group and liver cancer model group were (0.83±0.04), (0.85±0.05) and (74.38±7.18), respectively. MCP-1 expression levels in the control group, sham operation group and liver cancer model group were (2.74±0.21), (2.73±0.20) and (76.27±8.49), respectively. TGF-β expression levels in the control group, sham operation group and the liver cancer model group were (1.48±0.37), (1.47±0.36) and (5.98±1.12), respectively. IL-1α expression levels in the control group, sham operation group and liver cancer model group were (1.19±0.28),

(1.18±0.28) and (0.97±0.19), respectively. IL-1β expression levels in the control group, sham operation group and liver cancer model group were (1.87±0.35), (1.86±0.35), (2.10±0.24), respectively. The expressions of IL-6, MCP-1 and TGF-β in the liver cancer model group were significantly higher than those in the control group and the sham operation group and the differences were statistically significant (all $p<0.001$). However, there was no significant difference in the expression levels of IL-1α and IL-1β between the groups, and the differences were not statistically significant (all $p > 0.05$). There was no difference between the control group and the sham operation group ($p > 0.05$). See Figure 1 for details, which indicates the condition of IL-6, MCP-1, and TGF-β in liver cancer.

Expression Levels of Hepatocarcinoma Stem Cells Marker of Each Group in Tissues

The expressions of CD90 in the control group, sham operation group and liver cancer model group were (1.18±0.02), (1.19±0.02), (54.38±24.48), respectively. The expressions of CD133 in the control group, sham operation group and liver cancer model group were (5.37±1.27), (5.36±1.27), (25.47±8.39), respectively. The expressions of AFP in the control group, sham operation group and liver cancer model group were (1.38±0.14), (1.37±0.13), (1.45±0.28), respectively. The expression levels of CD90 and CD133 in the liver cancer model group were significantly higher than those in the control group and the sham operation group and the differences were statistically significant (all $p<0.001$). There was no significant difference in AFP among the groups and the difference was not statistically significant (all $p > 0.05$). There was no difference between the control group and the sham operation group (all $p > 0.05$). See Figure 2 for details, which shows CD90 and CD133 in liver cancer.

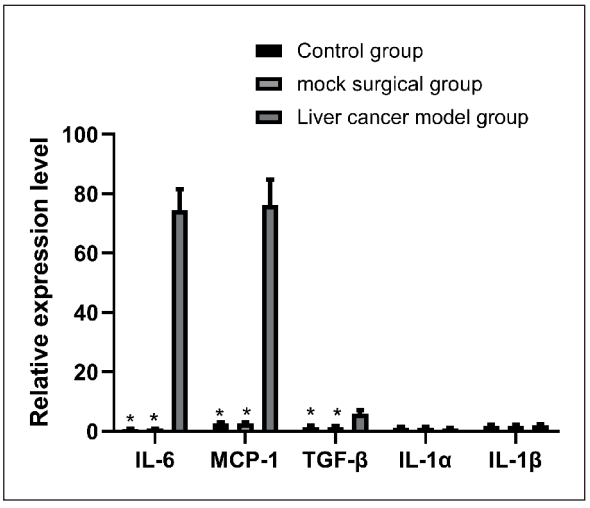

Figure 1. The expression levels of inflammatory factor in the tissue in the tissue. The expressions of IL-6, MCP-1, and TGF-β in the liver cancer model group were significantly higher than those in the control group and the sham operation group ($p<0.001$), while there was no significant difference in the expression levels of IL-1α and IL-1β between the groups ($p> 0.05$). There was no difference between the control group and the sham operation group ($p> 0.05$) Note: * indicates comparison with the liver cancer model group ($p<0.05$).

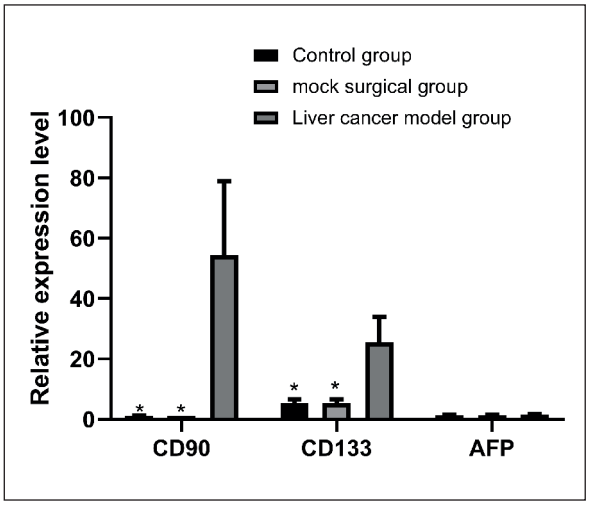

Figure 2. Expression levels of liver cancer stem cell markers in each group of tissues. The expression levels of CD90 and CD133 in liver cancer model group were significantly higher than those in control group and sham operation group. There was no significant difference in AFP between the groups ($p> 0.05$), and there was no difference between the control group and the sham operation group ($p> 0.05$). Note: * indicates comparison with liver cancer model group ($p <0.05$).

Relative Protein Expression Levels of Inflammatory Factors in Each Group

The relative protein expressions of IL-6 in the control group, sham operation group and liver cancer model group were (0.24 ± 0.03), (0.23 ± 0.02), (0.89 ± 0.05), respectively. The relative protein expressions of MCP-1 in the model group were (0.17 ± 0.02), (0.18 ± 0.02), and (0.43 ± 0.03), respectively. The relative protein expressions of TGF-β in the control group, sham operation group, and liver cancer model group were (0.24 ± 0.03), (0.23 ± 0.03), (0.62 ± 0.04), respectively. The relative protein expressions of IL-1α in the control group, sham opera-

tion group and liver cancer model group were (0.16 ± 0.02), (0.15 ± 0.02), (0.17 ± 0.03), respectively. The relative protein expressions of IL-1β in the control group, sham operation group and liver cancer model group were (0.21 ± 0.03), (0.22 ± 0.02), and (0.23 ± 0.03), respectively. The relative protein expressions of IL-6, MCP-1, and TGF-β in the liver cancer model group were significantly higher than those in the control group and the sham operation group (p <0.001), while there was no significant difference in the relative protein expression levels of IL-1α and IL-1β between groups (p> 0.05). There was no difference between the control group and the sham operation group (p> 0.05). Figure 3 presents the relative protein levels of IL-6, MCP-1 and TGF-β in liver cancer.

Relative Protein Expression Levels of Liver Cancer Stem Cell Markers in Each Group

The relative protein expressions of CD90 in the control group, sham operation group and liver cancer model group were (1.23 ± 0.21), (1.22 ± 0.21), and (3.24 ± 0.56) respectively. The relative protein expressions of CD133 were (0.35 ± 0.22), (0.36 ± 0.22), and (1.20 ± 0.47) respectively. The relative protein expressions of AFP in the control group, sham operation group, and liver cancer model group were (2.45 ± 1.03), (2.44 ± 1.03), (2.46 ± 1.02) respectively. The relative protein expression levels of CD90 and CD133 in liver cancer model group were significantly higher than those in control group and sham operation group (p<0.001). There was no significant difference in AFP between the groups (p> 0.05), and there was no difference between the control group and the sham operation group (p> 0.05). Figure 4 shows the relative protein levels of CD90 and CD133 in liver cancer.

Relationship Between Inflammatory Factors and Tumor Size and Tumor Weight

By observing the relationship between inflammatory factors, tumor size, tumor weight, it was concluded that inflammatory factors are closely related to tumors (p <0.05). It can be seen in Table II that inflammatory factors are closely related to tumors.

Cell Transfection

After transfection, related inflammatory factors were significantly decreased (p <0.05), indicating that transfection of inflammatory factor inhibitors was successful (Figure 5).

Observation of the Growth of Liver Cancer Cells at Different Time Periods

The growth of liver cancer cells was significantly inhibited after transfection with inhibitors (p <0.05; Figure 6) for details. It indicated that the growth of liver cancer cells was significantly inhibited after inflammatory factors were inhibited.

Observation of the Migration and Invasion of Liver Cancer Cells

The migration and invasion of liver cancer cells were significantly inhibited after transfec-

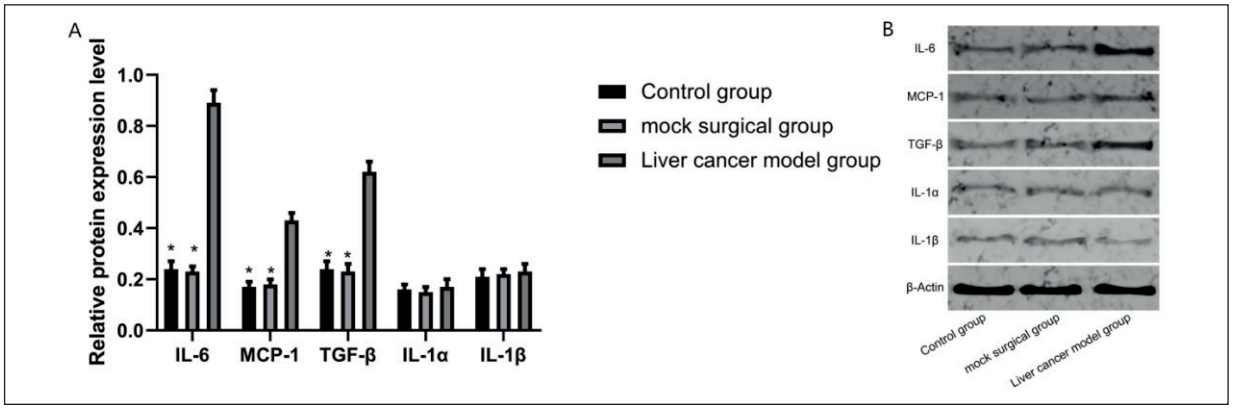

Figure 3. Relative protein expression levels of inflammatory factors in each group. **A,** The relative protein expressions of IL-6, MCP-1, and TGF-β in the liver cancer model group were significantly higher than those in the control group and the sham operation group (p<0.001), while there was no significant difference in the relative expression levels of proteins of IL-1α and IL-1βbetween the groups (p> 0.05). There was no difference between the control group and the sham operation group (p> 0.05). Note: * indicates comparison with the liver cancer model group (p<0.05). **B,** Western blot of relative protein expression levels of inflammatory factors in each group.

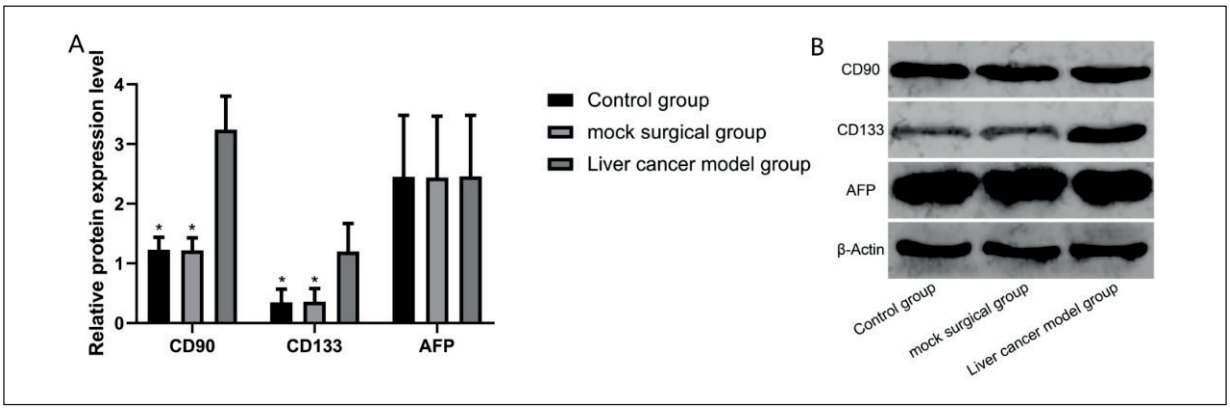

Figure 4. Relative protein expression levels of liver cancer stem cell markers in each group. **A,** The relative protein expression levels of CD90 and CD133 in the liver cancer model group were significantly higher than those in the control group and the sham operation group ($p<0.001$). There was no significant difference in AFP between the groups ($p> 0.05$), and there was no difference between the control group and the sham operation group ($p> 0.05$). Note: * indicates comparison with liver cancer model group ($p<0.05$). **B,** Western blot of relative expression levels of hepatoma stem cell markers in each group.

Table II. Relationship between inflammatory factors and tumor size and tumor weight.

	N	IL-6 (pg/ml)	*p*	MCP-1 (pg/ml)	*p*	TGF-β (pg/ml)	*p*
Tumor size (cm)			< 0.001		< 0.001		< 0.001
< 3	8	25.24 ± 3.52		63.24 ± 5.20		36.28 ± 3.59	
> 3	22	37.59 ± 4.29		78.13 ± 6.23		46.15 ± 4.77	
Tumor weight (g)			< 0.001		0.002		< 0.001
< 5	13	21.42 ± 2.67		56.13 ± 4.67		33.32 ± 3.25	
> 5	17	32.13 ± 3.24		62.14 ± 5.24		40.24 ± 4.53	

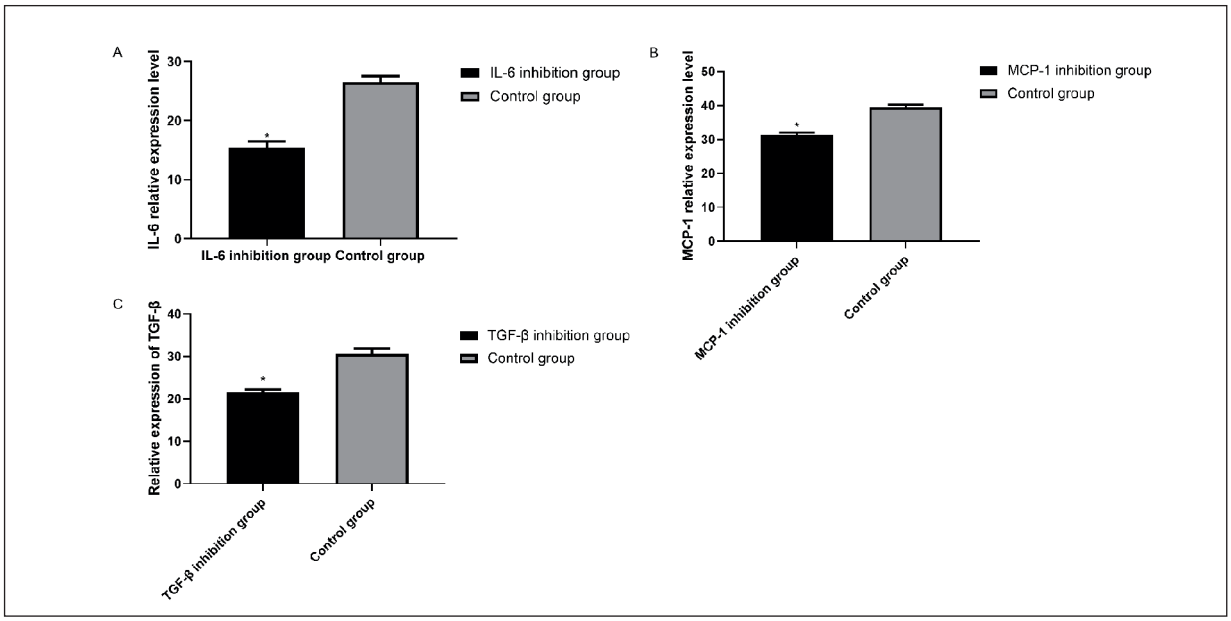

Figure 5. Transfection of cells. **A,** The relative expression of IL-6 in the IL-6 inhibition group was significantly lower than that in the control group ($p<0.05$). Note: *indicates comparison with the control group ($p<0.05$). **B,** The relative expression of MCP-1 in the MCP-1 inhibition group was lower than that in the control group ($p<0.05$). Note: *indicates comparison with the control group ($p<0.05$). **C,** The relative expression of TGF-β in the TGF-β inhibition group was lower than that in the control group ($p<0.05$). Note: *indicates comparison with the control group ($p<0.05$).

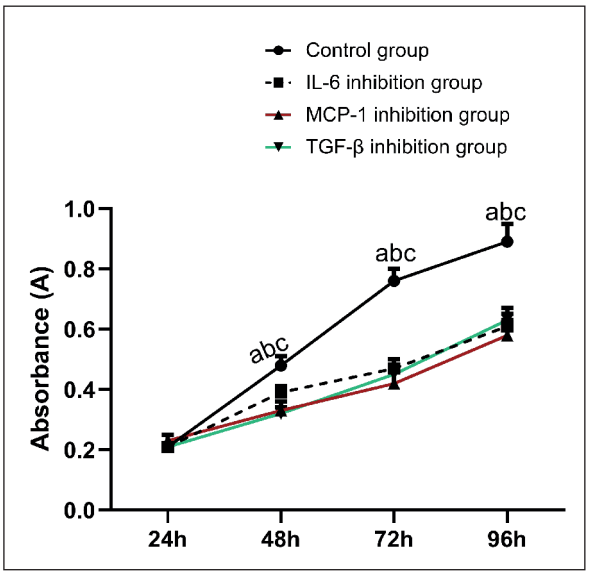

Figure 6. Growth of liver cancer cells at different time periods. The growth of hepatocellular carcinoma cells in the IL-6 inhibition group, MCP-1 inhibition group and TGF-β inhibition group was significantly inhibited (p<0.05). Note: a represents the comparison with the IL-6 inhibition group (p<0.05); b represents the comparison with MCP-1 inhibition group (p<0.05); c indicates comparison with TGF-β inhibition group (p<0.05).

tion with inhibitors (p <0.05). Figure 7 shows that the migration and invasion of hepatocellular carcinoma cells are significantly inhibited after inflammatory factors are inhibited.

Observation of the Apoptosis of Liver Cancer Cells

Apoptosis of HCC cells was significantly increased after transfection with inhibitors (p <0.05). It can be seen in Figure 8 that the inhibition of inflammatory factors promotes the apoptosis of liver cancer cells.

Correlation between Related Inflammatory Factors and Hepatocarcinoma Stem Cells Marker

The results showed that IL-6 was positively correlated with correlation of CD90 (r=0.835, p<0.05), MCP-1 was positively correlated with correlation of CD90 (r=0.677, p<0.05) and TGF-β was positively correlated with correlation of CD90 (r=0.766, p<0.05; Figure 9).

IL-6 was positively correlated with correlation of CD133 (r=0.699, p<0.05), MCP-1 was positively correlated with correlation of CD133 (r=0.700, p<0.05) and TGF-β was positively correlated with correlation of CD133 (r=0.772, p<0.05; Figure 10). The two figures show that inflammatory factors are closely related to liver cancer stem cell markers.

Discussion

Liver cancer remains one of the most prevalent and deadly cancer types in the world[17]. 782,000

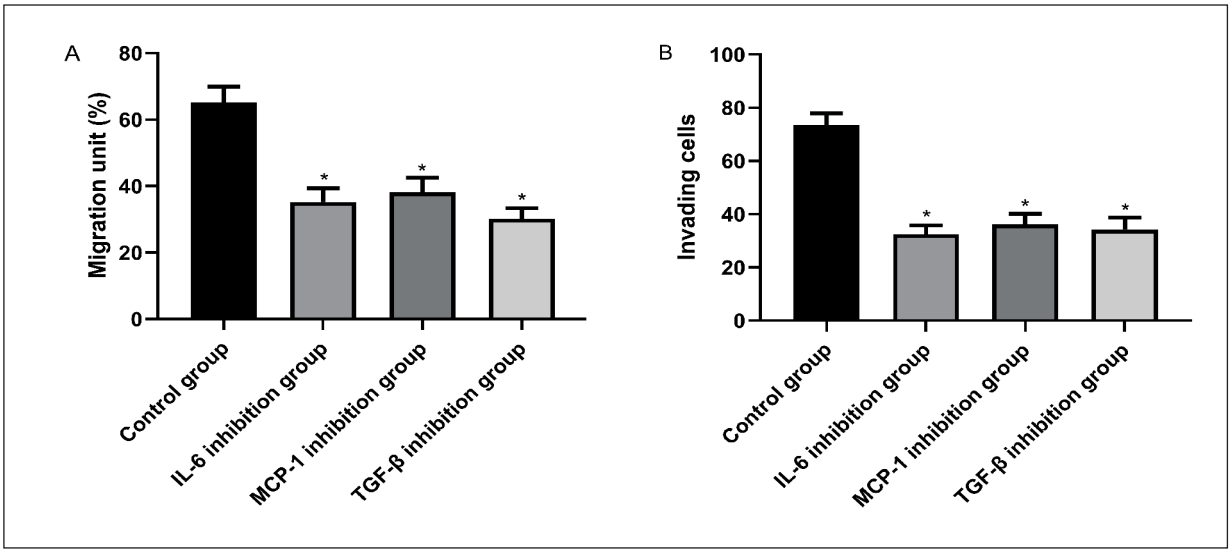

Figure 7. Migration and invasion of liver cancer cells. **A,** Hepatoma cell migration in the IL-6 inhibition group, MCP-1 inhibition group, and TGF-β inhibition group were significantly inhibited (p<0.05). Note: * indicates comparison with the control group (p<0.05). **B,** Invasion of hepatocellular carcinoma cells in the IL-6 inhibition group, MCP-1 inhibition group and TGF-β inhibition group was significantly inhibited (p<0.05). Note: * indicates comparison with the control group (p<0.05).

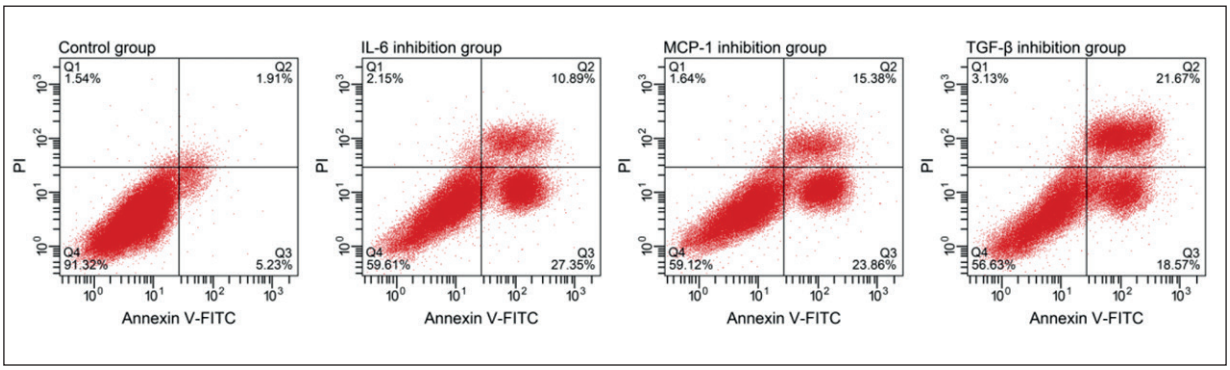

Figure 8. Apoptosis of liver cancer cells. Apoptosis of hepatocellular carcinoma cells in IL-6 inhibition group, MCP-1 inhibition group and TGF-β inhibition group increased significantly (p<0.05).

people are diagnosed with liver cancer each year and 746,000 people die from this disease, accounting for 5.6% of all new cancer cases and 9.1% of all cancer deaths worldwide[18]. The main cause of liver cancer is the infection of hepatotropic virus[19].

The IL-6 signaling pathway is harmful to the liver and may eventually lead to the development of liver tumors[20]. MCP-1 plays a key role in the recruitment and activation of monocytes during inflammation. Elevated serum level of MCP-1 in various cancer patients is associated with cancer progression. TGF-β is a central regulator of chronic liver disease and is involved in and contributes to all stages of the disease, from initial liver damage to inflammation and fibrosis to cirrhosis and hepatocellular carcinoma. In this study, IL-6, MCP-1, and TGF-β were abnormally up-regulated in liver cancer tissues, so we speculated that the up-regulation of inflammatory factors may be related to tumorigenesis. Therefore, inflammatory factors were inhibited from observing observe the biological effects on liver cancer. The results showed that the inhi-

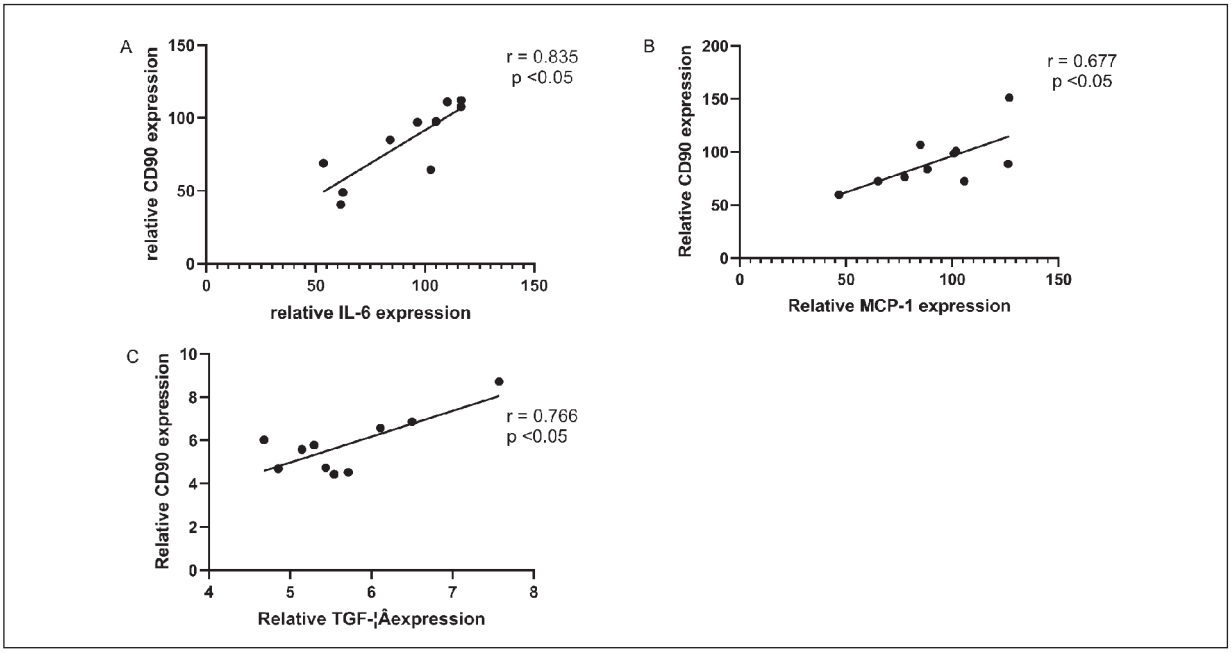

Figure 9. Correlation between inflammatory factors and CD90. **A,** Pearson correlation analysis between IL-6 and CD90. IL-6 and CD90 were positively correlated (r = 0.835, p<0.05). **B,** Pearson correlation analysis between MCP-1 and CD90. MCP-1 and CD90 were positively correlated (r = 0.677, p<0.05). **C,** Pearson correlation analysis between TGF-β and CD90. TGF-β and CD90 were positively correlated (r = 0.766, p<0.05).

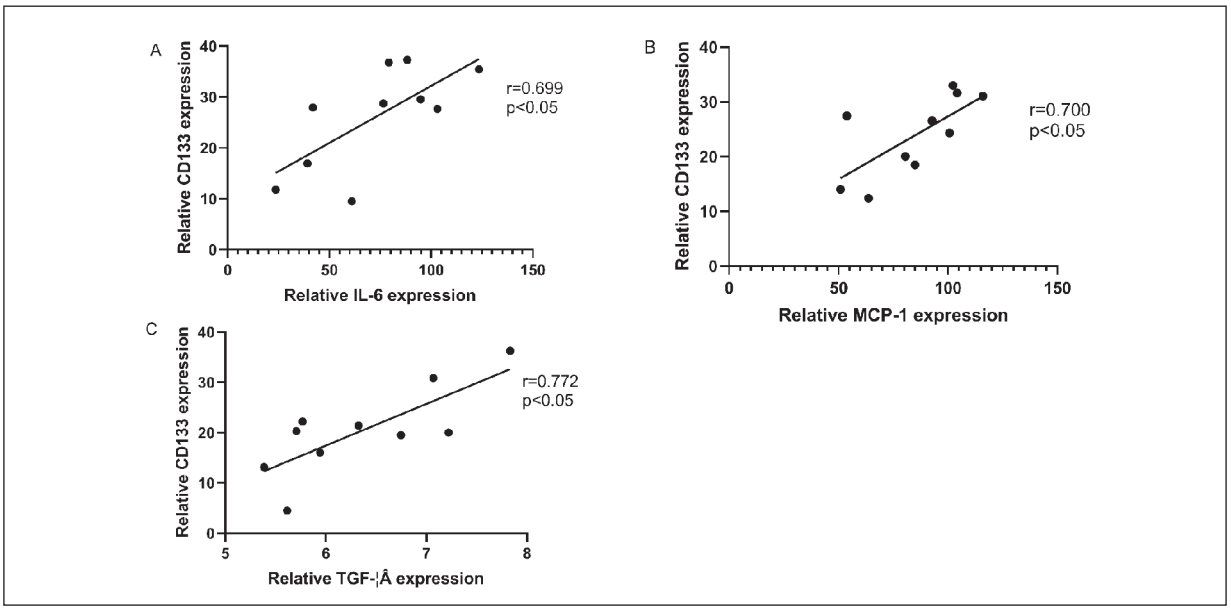

Figure 10. Correlation between inflammatory factors and CD133. **A,** Pearson correlation analysis between IL-6 and CD133. IL-6 and CD133 were positively correlated (r = 0.699, $p<0.05$). **B,** Pearson correlation analysis between MCP-1 and CD133. MCP-1 and CD133 were positively correlated (r = 0.700, $p<0.05$). **C,** Pearson correlation analysis between TGF-β and CD133. TGF-β and CD133 were positively correlated (r = 0.772, $p<0.05$).

bition of IL-6, MCP-1 and TGF-β significantly inhibited the growth of liver cancer cells and promoted apoptosis.

The inflammation is the body's response to harmful stimuli such as infectious, physiological or chemical stimuli. It releases various inflammatory mediators through immune cells, and inflammatory mediators can cause cell proliferation, genome instability, angiogenesis, anti-apoptosis, invasion and metastasis[21]. Inflammatory processes can regulate the progress of cancer, inhibiting or stimulating its growth. The activity of inflammatory cells and the type and level of inflammatory regulators affect the balance between their tumor-promoting and anti-tumor effects[22]. The tumor microenvironment is mainly coordinated by inflammatory cells and is an indispensable participant in the tumor process, promoting tumor cell proliferation, survival, and migration[23]. In some types of cancer, inflammatory conditions exist before the malignant changes[24]. All explain that inflammatory factors may be related to the occurrence and progression of liver cancer.

CD133 is a well-characterized cancer stem cell marker that is involved in tumor cell proliferation, metastasis, tumorigenesis and recurrence, and chemical and radioresistance. CD90 is a marker of hepatocellular carcinoma stem cells with tumorigenic activity[25], and is found in blood samples from 91.6% of liver cancer patients[26]. In this study, it was found that CD90 and CD133 were upregulated in liver cancer. Pearson correlation analysis was used to analyze the relationship between relevant inflammatory factors and liver cancer stem cell markers, and the results showed that inflammatory factors were positively correlated with liver cancer stem cell markers. According to previous study, IL-6-mediated signal transduction enhances CD133 expression and promotes the progression of hepatocellular carcinoma[27]. Moreover, it is suggested that there may be a close relationship between IL-6 and CD133. In a study by Rawal et al[28], secretory factors such as TGF-β from leading endothelial cells may enhance the expression of CD133 and provide an aggressive epithelial-mesenchymal transition phenotype for hepatitis B virus-induced hepatitis B protein-infected liver cancer cells. This indicates a close correlation between inflammatory factors and liver cancer stem cell markers. Therefore, we speculated that the up-regulation of inflammatory factors activated liver cancer stem cell markers, thereby promoting the occurrence and progression of liver cancer. However, the specific mechanism has not yet been known.

Conclusions

In summary, inflammatory factors in rats with induced liver cancer are closely related to liver cancer stem cell markers. Inflammatory factors may also have an effect on the biological mechanism of liver cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siddiqui H, Al-Ghafari A, Choudhry H, Al Doghaither H. Roles of long non-coding RNAs in colorectal cancer tumorigenesis: a review. Mol Clin Oncol 2019; 11:167-172.
2) Ji J, Shi J, Budhu A, Yu Z, Forgues M, Roessler S, Ambs S, Chen Y, Meltzer PS, Croce CM, Qin LX, Man K, Lo CM, Lee J, Ng IO, Fan J, Tang ZY, Sun HC, Wang XW. MicroRNA expression, survival and response to interferon in liver cancer. N Engl J Med 2009; 361: 1437-1447.
3) Affo S, Yu LX, Schwabe RF. The role of cancer-associated fibroblasts and fibrosis in liver cancer. Annu Rev Pathol 2017; 12: 153-186.
4) Petrick JL, Braunlin M, Laversanne M, Valery PC, Bray F, McGlynn KA. International trends in liver cancer incidence, overall and by histologic subtype, 1978-2007. Int J Cancer 2016; 139: 1534-1545.
5) Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.
6) Raucci MG, Fasolino I, Caporali M, Serrano-Ruiz M, Soriente A, Peruzzini M, Ambrosio L. Exfoliated black phosphorus promotes in vitro bone regeneration and suppresses osteosarcoma progression through cancer-related inflammation inhibition. ACS Appl Mater Interfaces 2019; 11: 9333-9342.
7) Ramirez MU, Stirling ER, Emenaker NJ, Roberts DD, Soto-Pantoja DR. Thrombospondin-1 interactions regulate eicosanoid metabolism and signaling in cancer-related inflammation. Cancer Metastasis Rev 2018; 37: 469-476.
8) Xiong S, Wang R, Chen Q, Luo J, Wang J, Zhao Z, Li Y, Wang Y, Wang X, Cheng B. Cancer-associated fibroblasts promote stem cell-like properties of hepatocellular carcinoma cells through IL-6/STAT3/Notch signaling. Am J Cancer Res 2018; 8: 302-316.
9) Knoll P, Schlaak J, Uhrig A, Kempf P, Meyer zum Büschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. J Hepatol 1995; 22: 226-229.
10) Chen S, Hoffman RA, Scott M, Manson J, Loughran P, Ramadan M, Demetris AJ, Billiar TR. NK1.1+ cells promote persistent tissue damage and inflammation after traumatic hemorrhagic shock. J Leukoc Biol 2017; 102: 127-134.
11) Adizie JB, Khakwani A, Beckett P, Navani N, West D, Woolhouse I, Harden SV. Stage III Non-small Cell Lung Cancer Management in England. Clin Oncol (R Coll Radiol) 2019; 31: 688-696.
12) Zhang S, Yang X, Wang L, Zhang C. Interplay between inflammatory tumor microenvironment and cancer stem cells. Oncol Lett 2018; 16: 679-686.
13) Xiang X, Deng L, Xiong R, Xiao D, Chen Z, Yang F, Liu K, Feng G. Tex10 is upregulated and promotes cancer stem cell properties and chemoresistance in hepatocellular carcinoma. Cell Cycle 2018; 17: 1310-1318.
14) Moazen B, Zarrinhaghighi A, Nejatollahi F. Selection and evaluation of specific single chain antibodies against CD90, a marker for mesenchymal and cancer stem cells. Rep Biochem Mol Biol 2018; 7: 45-51.
15) Wang K, Sun D. Cancer stem cells of hepatocellular carcinoma. Oncotarget 2018; 9: 23306-23314.
16) Xiao B, Cui LM, Ma DJ, Liu SP, Zhang XW. Endoplasmic reticulum stress in diethylnitrosamine-induced rat liver cancer. Oncol Lett 2014; 7: 23-27.
17) Ahn YH, Shin PM, Kim YS, Oh NR, Ji ES, Kim KH, Lee YJ, Kim SH, Yoo JS. Quantitative analysis of aberrant protein glycosylation in liver cancer plasma by AAL-enrichment and MRM mass spectrometry. Analyst 2013; 138: 6454-6462.
18) Zuo TT, Zheng RS, Zhang SW, Zeng HM, Chen WQ. The incidence and mortality of liver cancer in China in 2011. Chin J Cancer 2015; 34: 508-513.
19) Mehta A, Herrera H, Block T. Glycosylation and Liver Cancer. Adv Cancer Res 2015; 126: 257-279.
20) Schmidt-Arras D, Rose-John S. IL-6 liver pathway: from pathophysiology to treatment. J Hepatol 2016; 64: 1403-1415.
21) Shrihari TG. Dual role of inflammatory mediators in cancer. Ecancermedicalscience 2017; 11: 721.
22) Korniluk A, Koper O, Kemona H, Dymicka-Piekarska V. From inflammation to cancer. Ir J Med Sci 2017; 186: 57-62.
23) Coussens LM, Werb Z. Inflammation and cancer. Nature 2002; 420: 860.
24) Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436.
25) Luo J, Wang P, Wang R, Wang J, Liu M, Xiong S, Li Y, Cheng B. The Notch pathway promotes the characteristics of cancer stem cells in CD90+ cells in hepatocellular carcinoma. Oncotarget 2016; 7: 9525.

26) Yang R, An LY, Miao QF, Li FM, Han Y, Wang HX, Liu DP, Chen R, Tang SQ. Effective elimination of liver cancer stem-like cells by CD90 antibody targeted thermo sensitive magneto liposomes. Oncotarget 2016; 7: 35894-35916.

27) Lee SO, Yang X, Duan S, Tsai Y, Strojny LR, Keng P, Chen Y. IL-6 promotes the growth and epithelial-mesenchymal transition of CD133+ cells in non-small cell lung cancer. Oncotarget 2016; 7: 6626.

28) Rawal P, Siddiqui H, Hassan M, Choudhary MC, Tripathi DM, Nain V, Trehanpati N, Kaur S. Endothelial cell-derived TGF-β promotes epithelial-mesenchymal transition of CD133 in hepatoma cells infected with HBx. Front Oncol 2019; 9: 308.