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Original Article

Effect of interleukin-6 on invasion and migration of hepatic carcinoma cells

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Abstract: Objective: To investigate the effect of Interleukin-6 on invasion and migration of the human HepG2 cells. Method: The HepG2 cells were treated by human IL-6, and Transwell chamber assay and wound scratch assay were used to test the cell invasion and migration respectively; the expression of STAT3, p-STAT3, RhoA and ROCK kinase proteins were detected by Western blotting; tumor was formed subcutaneously in nude mice and the tumor-bearing mice were treated by human recombinant IL-6, to observe the survival time and the changes in tumor volume in the nude mice. Results: At a concentration of 25 ng/ml for IL-6, proliferation or migration of the HepG2 cells were significantly enhanced; human recombinant IL-6 could promote the invasion and migration of the HepG2 cells; after the cells were treated by human recombinant IL-6, the levels of p-STAT3, RhoA and ROCK kinase proteins were significantly increased, the tumor growth in tumor-bearing mice was significantly increased and the survival time was significantly shortened. Conclusion: IL-6 can activate STAT3 to increase the expression levels of RhoA and ROCK, promote cytoskeletal remodeling, and thus promote invasion and migration of the HepG2 cells.

Keywords: Interleukin-6, STAT3, migration, hepatic carcinoma

Introduction

Hepatic carcinoma is one of the most common malignant tumors worldwide and also the third largest lethal cancer in the world. Each year, the number of new cases with hepatic carcinoma in China accounts for over 50% of the total number in the world, and over 150,000 people die of hepatic carcinoma, accounting for over 40% worldwide [1]. The most common cause of hepatic carcinoma is the hepatitis virus infection, and other risk factors include aflatoxin, alcohol, α-antitrypsin deficiency, etc. Although the survival rate of patients with hepatic carcinomas is greatly increased with introduction and application of surgical resection, radiofrequency ablation, liver transplantation and targeted therapy, recurrence and metastasis may occur in more than half of the patients within five years. Numerous studies have shown that [2-4] most patients with hepatic carcinoma have micrometastasis before treatment, which becomes the direct cause of metastasis and recurrence of hepatic carcinoma.

Tumor metastasis is a complex multi-step, multi-stage process, mainly including local infiltration, invasion in blood vessels, metastasis with and survival in the blood circulation system, emigration from blood vessels, and settlement and proliferation in new sites, and the cytoskeletal changes, cell adhesion and changes in kinetic characteristics, epithelial-mesenchymal transition and activation of various signaling pathways were involved [5]. Therefore, identifying the HCC metastasis-related cytokines has great significance for elucidating the mechanism of metastasis of hepatic carcinoma, and has important clinical and scientific values for evaluating the treatment of hepatic carcinoma and screening of related drugs.

Interleukin-6 (IL-6) is a T cell-derived pleiotropic cytokine that induces proliferation, differentiation, maturation of B lymphocytes and produces antibodies [6]. It regulates the body immune system, stimulates the hematopoietic system, regulates liver regeneration, induces protein synthesis in the liver in the acute phase, and induces responses in the acute stage, etc. [7]. In patients with tumor, IL-6 is produced in the inflammatory cells and stromal cells in tumor microenvironment. IL-6 is highly expressed in colon cancer, ovarian cancer, cholangiocellular

carcinoma, etc. Studies have shown that [8, 9] IL-6 was expressed in high level in patients with hepatic carcinoma, and the elevated IL-6 level is associated with poor prognosis. In this study, we investigated the effects of IL-6 on invasion and migration of the HepG2 cells, and explored the related mechanism.

Materials and methods

Cell line and main reagents

Human HCC line HepG2 was purchased from ATCC. Cell culture conditions: cultured in RPMI containing 10% fetal calf serum at 37°C, 5% CO_2. The fetal calf serum and RPMI 1640 medium were purchased from Gibco Corp. IL-6R, STAT3, p-STAT3, ROCK kinase, and RhoA rabbit monoclonal antibodies were purchased from Abcam (ab83053, ab68153, ab76315, ab45-171 and ab187027). Human recombinant IL-6 was purchased from PeproTech Corp. Transwell chambers were purchased from BD Biosciences. BALB/c (nu/nu) mice were provided by the Animal Experiment Center, Zhengzhou University, 4 to 6 weeks of age, weighed 15-20 g, male; raised in a SPF-level environment. This study was approved by the Ethics Committee of the Zhumadian People hospital.

Preparation of IL-16

100 μg of human recombinant IL-6 was dissolved in 1 ml DMSO (Dimethylsulfoxide, DMSO), to prepare into the concentrations of 5, 10, 25, 50 ng/ml.

Expression of IL-6R detected by western blotting

Two hours after treatment of the HepG2 cells by human recombinant IL-6 at different concentrations, the total proteins were extracted, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 10% SDS-PAGE was prepared, and 20 μg protein sample was added into each hole, then transferred to a PVDF membrane using the electric wet transfer method, sealed 2h with 5% skim milk, and the primary antibody was diluted by 1:1000 TBST (IL-6R), overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. In the experiment, the HepG2 cells treated with the human recombinant IL-6 were in the experiment group, and the untreated HepG2 cells were in the control group. The experiment was performed in triplicate.

Changes in hepatic carcinoma cell invasion ability after treatment with human recombinant IL-6 by Transwell invasion assay

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 μl (0.2 μg/μl) Matrigel gel was evenly applied to inner membrane of Transwell chamber, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, cells were diluted with 2.5×10^4/mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 200 μL each well, and 500 μL of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with formalin, stained by crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields (×40). The experiment was performed in triplicate.

Changes in hepatic carcinoma cell migration ability after treatment with human recombinant IL-6 by wound scratch assay

Wound scratch assay: The HepG2 cells were inoculated to a 6-well plate, and when cell confluence reached 90%, scratch from up to bottom using a 200 μl sterile pipette tip, observe under a microscope, to measure the initial distance of scratch (0 time); at 24 h, 48 h and 72 h, the distances of scratch were measured respectively and photographed, to calculate the cell migration rate. The experiment was performed in triplicate.

Expression of STAT3, p-STAT3, ROCK kinase and RhoA detected by western blotting after treatment of the HepG2 cells with human recombinant IL-6

Two hours after treatment of the HepG2 cells by human recombinant IL-6 at different concentrations, the total proteins were extracted, and the protein concentrations were determined by BCA method, and then loading buffer was

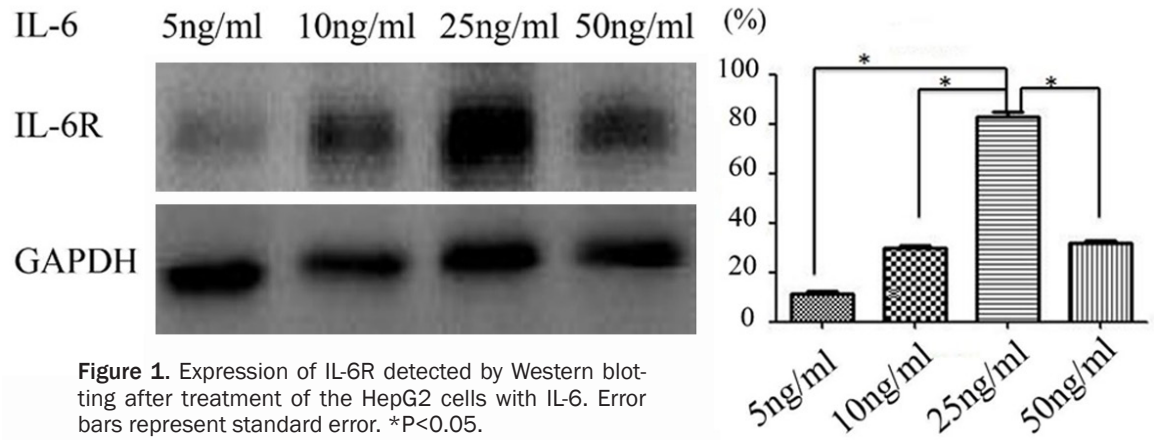

Figure 1. Expression of IL-6R detected by Western blotting after treatment of the HepG2 cells with IL-6. Error bars represent standard error. *P<0.05.

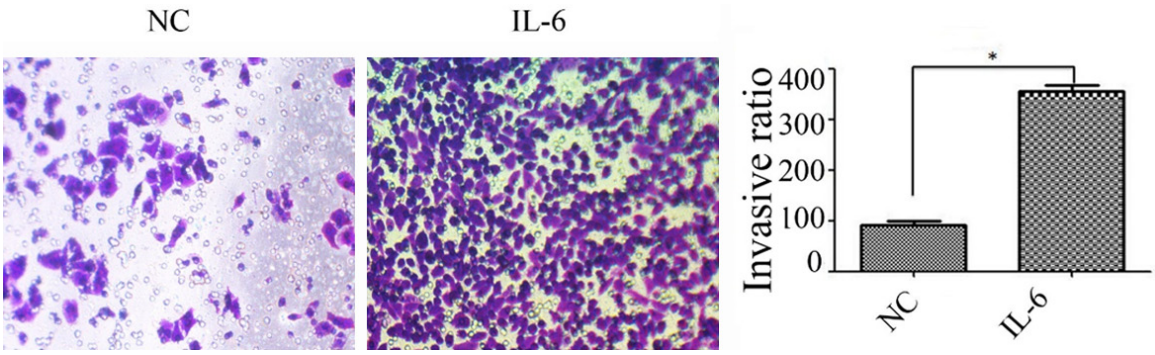

Figure 2. Effect of IL-6 on the invasion ability of the HepG2 cells detected by Transwell invasion assay. Error bars represent standard error. *P<0.05.

added for protein denaturation. 8%~10% SDS-PAGE was prepared, and 20 μg protein sample was added into each hole, then transferred to a PVDF membrane using the electric wet transfer method, sealed 2 h with 5% skim milk, and the primary antibody was diluted by 1:1000 TBST (STAT3, p-STAT3, ROCK kinase and RhoA), overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

Effects of human recombinant IL-6 on tumor growth and metastasis observed by tumor formation in nude mice

The HepG2 cells in the logarithmic growth phase was digested in a culture flask, to adjust the concentration at 2×10^8/ml. 0.1 ml of cell suspension was taken and injected to the left forelimb armpit subcutaneously of each nude mouse, a total of 10 mice. The tumor growth conditions of the nude mice were observed every day. After inoculation for one week, the tumor grew to about 5-6 mm. The nude mice were divided into 2 groups, 5 mice each group. (1) Experimental group: the tumor-bearing nude mice were given 100 μl of 1 μg/100 μl human IL-6 solution, injected intraperitoneally from the right side, once daily, for three consecutive days. (2) Control group: treated with 100 μl saline, injected intraperitoneally from the right side, once daily, for three consecutive days. The tumor diameter (a) and the vertical orthogonal diameter (b) of tumor-bearing mice were measured every other day. The tumor size was calculated as $V\ (mm^3)=a\times b^2/2$.

Statistical analysis

The SPSS 19.0 software was used for statistical analysis, measurement data were expressed in ($\overline{x}\pm s$), t-test was employed for comparison of means between groups, and P<0.05 indicated statistically significant difference.

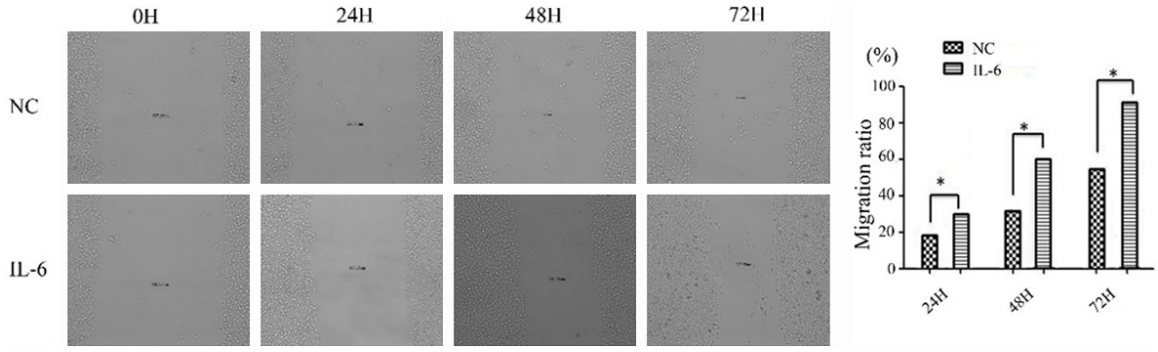

Figure 3. Effect of IL-6 on the migration ability of the HepG2 cells detected by wound scratch assay. Error bars represent standard error. *P<0.05.

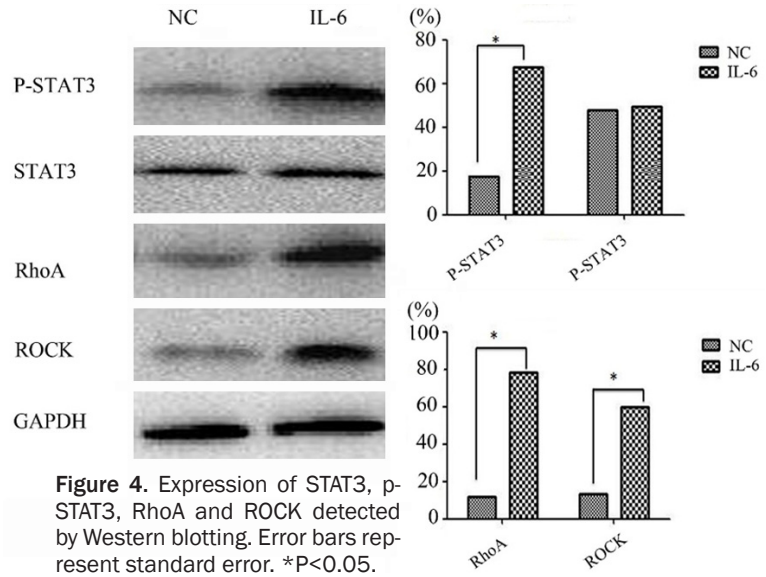

Figure 4. Expression of STAT3, p-STAT3, RhoA and ROCK detected by Western blotting. Error bars represent standard error. *P<0.05.

Results

Up-regulation of IL-6R expression in the HepG2 cells by human recombinant IL-6

IL-6R is the receptor of IL-6, the role of which is largely determined by its structure and functions. By detecting the expression of IL-6R, we identified the optimal concentration of human recombinant IL-6 for treating the HepG2 cells. The Western blotting results showed that (**Figure 1**) with the increase in IL-6 concentration, the expression of IL-6R increased gradually, and it was highest when the concentration was 25 ng/ml, then with increase in concentration, the expression of IL-6R decreased gradually [(82.32±4.58)% vs (8.80±1.16)%, (28.46±2.27)%, (27.84±2.23)%, P<0.05], suggesting that 25 ng/ml was the optimal concentration of human recombinant IL-6 for treating the HepG2 cells.

Invasion of the HepG2 cells promoted by human recombinant IL-6

The ability of cells to pass through the Matrigel gel reflects their invasion ability. Transwell results showed (**Figure 2**) that the number of cells passing through the gel was 89.57±6.76 in the control group, significantly less than that in the experiment group (383.92±16.88), with statistically significant difference (P<0.05). This showed that human recombinant IL-6 could promote invasion of the HepG2 cells.

Migration of the HepG2 cells promoted by human recombinant IL-6

The width of scratches in any three parts of cells in each group was measured under a microscope at the time points of 0 h, 24 h, 48 h and 72 h. The migration rate was calculated according to the formula: Migration rate=$[D_{(t=24\ h,\ 48\ h,\ 72\ h)}-D_{(t=0\ h)}]/D_{(t=0\ h)}$. Results of the wound scratch assay (**Figure 3**) showed that compared with the control group, at 24 h, 48 h and 72 h, migration rate in the experiment group increased significantly [24 h (26.46±2.48)% vs (18.03±1.91)%, P<0.05; 48 h (58.53±4.76)% vs (30.15±2.74)%, P<0.05; 72 h (86.25±6.93)%

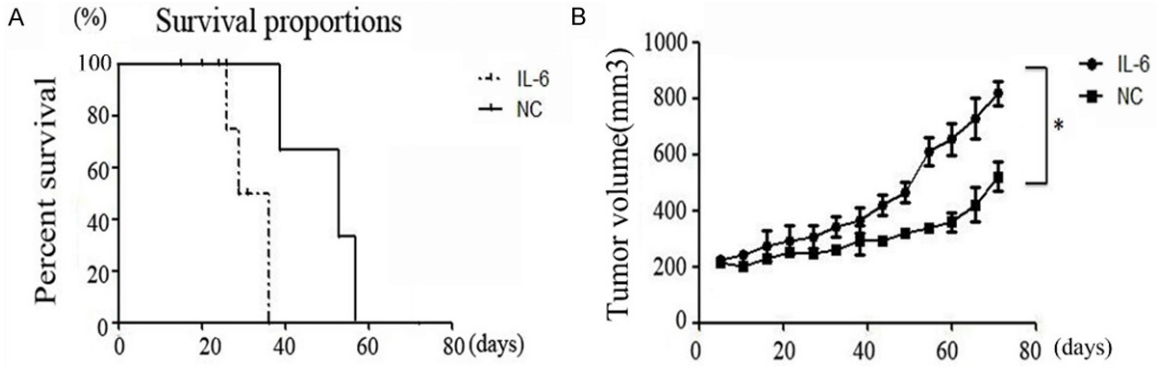

Figure 5. Effect of IL-6 on tumor growth investigated in experiment *in vivo*. A. Comparison of the survival time of nude mice. B. Comparison of the size of the tumor growth in nude mice.

vs (55.62±4.04)%, P<0.05], with statistically significant difference. This showed that human recombinant IL-6 could promote migration of the HepG2 cells.

Expression of cytoskeleton-associated proteins in the HepG2 cells promoted by human recombinant IL-6 through activation of STAT3

Cell invasion and migration is associated with the change in cell adhesion, degradation of extracellular matrix and the morphological changes, and all these processes are completed via constantly changing cytoskeletons [10]. The experiment described above showed that human recombinant IL-6 could promote invasion and migration of the HepG2 cells. Thus, it could be inferred that human recombinant IL-6 might induce cytoskeleton reconstruction by altering the cytoskeleton, thereby leading to cell invasion and migration. Rho protein family is an important regulatory protein of cytoskeleton actin [11]. We speculate that human recombinant IL-6 may activate the signaling pathways of associated cytoskeleton by regulating the Rho family proteins, to promote the invasion and migration of hepatic carcinoma cells.

The transcription factor STAT3 (signal transducer and activator of transcription) plays a critical role in signal transduction and activation of transcription. Many cytokines including interleukins, growth hormones, epidermal growth factor, platelet-derived factor and interferon function through the STAT signaling pathway [12]. Studies have shown that persistent activation of STAT3 is an important reason of tumor proliferation and metastasis [13], therefore, we inferred that human recombinant IL-6 could influence the cytoskeleton function by activating STAT3, thereby promoting invasion and migration of hepatic carcinoma cells.

The Western blotting results (**Figure 4**) showed that compared with the control group, the expression level of p-STAT3 in the experiment group increased significantly [(68.62±3.92)% vs (19.09±1.19)%, P<0.05]; compared with the control group, the expression level of STAT3 in the experiment group showed no significant changes [(44.51±3.11)% vs (45.61±3.19)%, P>0.05]; compared with the control group, the expression levels of RhoA and ROCK in the experiment group increased significantly [RhoA (79.26±4.17)% vs (9.21±1.11)%; ROCK (60.19±3.52)% vs (10.60±1.30)%, P<0.05]. This suggested that human recombinant IL-6 could cause cytoskeleton reconstruction and thus promote invasion and migration of the HepG2 cells by activating STAT3 and increasing the expression levels of RhoA and ROCK.

Subcutaneous tumor formation experiment in nude mice showed that human recombinant IL-6 could promote tumor growth and shorten the survival time of nude mice

The survival time (**Figure 5A**) of tumor-bearing mice was 4-8 weeks, with a median of 6 weeks. Tumor growth could be seen in the left armpit of the mice by autopsy, and the tumors were grey white, solid, round or elliptical, with nodular projection on the surface and fish flesh-like section. The tumor formation rate is 100%.

Tumor growth in nude mice (**Figure 5B**): tumors in nude mice of the experiment group and the control group increased gradually, but the

growth rate of tumor in the experiment group was higher than the control group, with statistically significant difference (P<0.05).

Survival time of nude mice: compared with the control group, the mean survival time of nude mice in the experiment group was significantly shortened [(28.6±2.8)% vs (53.3±3.4)%, P<0.05].

Discussions

IL-6 is one of the cytokines with the most comprehensive functions. Both the lymphocytes and non-lymphocytes can secrete IL-6, and many antigens and non-antigenic materials can also stimulate and induce the secretion of IL-6, e.g., the mutual induction and mutual regulation between bacterial endotoxin or other cell factors. The IL-6 receptor system is composed of two kinds of membrane proteins: ligand-binding chain IL-6R binding with IL-6, and the non-ligand-binding chain glycoprotein 130 (gp130) as the signal transducer. Gp130 is expressed in most of the tissues, and is involved in regulation and mediation of the signaling pathways related to a variety of cytokines [14]. The biological functions of IL-6 are as follows: it firstly activates IL-6R to form the soluble IL-6 receptor (soluble form of IL-6R) and then form the IL-6/sIL-6R complex, and activates gpl30 on the cell membrane surface, thereby mediating various signaling pathways and influencing the biological behaviors.

Initially, IL-6 is used as an inflammatory factor in most studies since it has a dual pro-inflammatory and anti-inflammatory role. Later, however, IL-6 was found to be associated with the size, metastasis, prognosis and survival rate of many malignant tumors. Kozłowski *et al.* [16] detected the serum IL-6 levels in 109 patients with breast cancer and 29 healthy subjects, and found out that the IL-6 expression in patients with gastric cancer was significantly increased and correlated with the staging of gastric cancer, moreover, it was consistent with the histopathological results. The study by V Michalaki *et al.* [17] showed that IL-6 was possibly correlated with severity of pancreatic cancer, which could be used as an indicator for monitoring the progression of pancreatic cancer and the therapeutic effects, together with other indicators. The study by Tchirkov *et al.* [18] revealed that the expression of glioma IL-6 in glioma was significantly higher than in other brain tissues. The high expression of IL-6 was positively correlated with poor prognosis of the patients.

IL-6 is also closely correlated with tumor metastasis. The study by JY Blay *et al.* [19] showed that the serum IL-6 level could be used a prognostic indicator for metastatic renal carcinoma. The study by GJ Zhang *et al.* [20] revealed that serum IL-6 was associated with progression and prognosis of metastatic breast cancer. The study by SF Shariat *et al.* [21] showed that serum IL-6 was associated with progression and prognosis of pancreatic cancer. After activating gp130, IL-6 induces activation of STAT3. The activated STAT3 molecule forms a dimer to transfer to the nucleus, and by binding with the target gene, it modifies expression of the gene and is involved in the processes including cell invasion and migration. In this study, by treating the HepG2 cells with human recombinant IL-6, the effects of IL-6 on invasion and migration of the hepatic carcinoma cells were investigated. It was shown in the study that treatment of the HepG2 cells with human recombinant IL-6 could promote the invasion and migration ability of the cells. In addition, the expression of p-STAT3 in the HepG2 cells after treatment with human recombinant IL-6 was significantly increased, suggesting that IL-6 could promote invasion and migration of the cells by activating STAT3.

Tumor infiltration and metastasis is a complicated process with multiple steps. The first step is to reduce the adhesive capacity of tumor cells, enhance cell mobility and infiltrate the peripheral tissues for distant metastasis. The key for realization ofadhesive capacity, mobility and morphological changes of the cells is recombination of microfilament cytoskeleton [22, 23]. Results from this study showed that after the HepG2 cells was treated with human recombinant IL-6, the expression levels of RhoA and ROCK kinase proteins were significantly increased, which suggested that IL-6 could promote invasion and migration of the cells by promoting cytoskeleton reconstruction.

In this study, by treating the HepG2 cells with human recombinant IL-6, the effects of IL-6 on invasion and migration of hepatic carcinoma cells were investigated in experiments *in vivo* and *in vitro*. It was shown in the study that IL-6

could induce cytoskeleton reconstruction by activating the STAT3 signaling pathway, thus promoting invasion and migration of the HepG2 cells. It suggested that IL-6 played an important part in progression and metastasis of hepatic carcinoma, and it might become a protein marker for predicting progression and prognosis of the disease and the therapeutic effect.

Disclosure of conflict of interest

None.

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