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Original Article

Ophiopogonin Binduceshepatocellular carcinoma MHCC97-H cell apoptosis and decreases invasion through inhibition of the JAK2/STAT3 signaling pathway

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Abstract: Ophiopogonin B (Op-B) is a bioactive component of Radix *Ophiopogon japonicus*, which is often used in traditional Chinese medicine to treat cancer. The present study aimed to investigate the effects of Op-B on human hepatocellular carcinoma MHCC97-H cells. Cell viability was assessed by CCK-8 assay. Cell cycle and apoptosis were measured with flow cytometry, respectively. Matrigel invasion assay was performed to detect the cell invasion of MHCC97-H cells induced by Op-B. Finally, Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting were used to explore the mechanism. The results demonstrated that high concentrations of Op-B (5, 10 and 20 μM) exerted potent anti-proliferative effects on MHCC97-H cells in a dose-dependent manner. Furthermore, cell cycle was arrestedand apoptotic rates and cell invasion were increased following treatment with Op-B. Next, we examined the JAK2/STAT3 signaling pathway and found that Op-B inhibited phosphorylation of JAK2 and STAT3. RT-qPCR and Western blotting indicated that treatment with Op-B increased the mRNA and protein expression levels of Caspase-3 and Bax, whereas the expression levels of Bcl-2, PCNA, MMP-2, VEGF and VEGFR2 were decreased. Additionally, IL-6 treatment not only activated STAT3 but also inhibited Op-B-induced decreases in the expression of PCNA, Bcl-2, MMP-2, VEGF, and VEGFR2 and increases in the expression of Bax and Caspase-3. Therefore, inhibition of JAK2/STAT3 signaling pathway may correlate with Op-B-mediated suppression of MHCC97-H cell viability and invasion.

Keywords: Ophiopogonin B, MHCC97-H cell, JAK2/STAT3, apoptosis, invasion

Introduction

Hepatocellular carcinoma (HCC), accounts for between 85% and 90% of primary liver cancers, is one of the most common malignant tumors worldwide and is the third leading cause of cancer-related deaths in the world, especially in Asia and Africa. The incidence of HCC was high enough, and the risk factors of HCC have emerged for hepatitis B virus, hepatitis C virus and autoimmune hepatitis [1, 2]. The prognosis of patients with HCC continues to be dismal because most patients with the disease are usually diagnosed at an advanced stage with severe intrahepatic and extrahepatic metastases [3, 4], despite improving treatment approaches, with a 5-year survival rate worldwide less than 5% [5]. As so, to explore the mechanism that underlies the HCC tumorigenesis is extremely important for its prevention and therapy. Research regarding the development of drugs for the treatment of HCC and its complications has recently gained attention. Chemoradiotherapy with its great toxicity causes a series of side effects such as nausea, vomiting and diarrhea, which severely damage to liver function [6]. Therefore, the identification of novel therapeutic strategies is essential to improve the clinical management of patients with HCC.

Ophiopogon japonicus (Thunb.) Ker-Gawl, widely distributed in South-east Asia, is a traditional Chinese medicine used to treat inflammatory and cardiovascular diseases, and has been reported to exert pharmacological effects, including anti-inflammatory, anti-arrhythmia, antibacteria, diuresis and microcirculation improvement [7, 8]. Ophiopogonin B (Op-B), which is isolated from the traditional Chinese herb Radix

O. japonicas, has been confirmed in various experiments as having anticancer effect. Op-B-induced apoptosis of SGC-7901 cells was associated with loss of MMP and increased ROS generation, and the anticancer mechanisms of Op-B may through regulating JNK1/2 and ERK1/2 signaling pathways [9]. Op-B induced autophagy and apoptosis in non-small cell lung cancer (NSCLC) cell lines NCI-H157 and NCI-H460 via inhibition of the PI3K/Akt/mTOR signaling pathway [10]. However, the role of Op-B in HCC has yet to be explored.

It is known that the dysregulation of the JAK/STAT signaling pathway may cause immunodeficiencies and cancers [11, 12]. One STAT family member, STAT3, is constitutively active in many human cancer cells and that, involved in cell proliferation, differentiation and apoptosis [11], the phosphorylation is mediated through the activation of JAK2 [12]. Recently many studies showed the important roles of VEGFR2 in potential drug discovery and molecular mechanism research [13, 14]. In addition, increasing evidence shows that within VEGFR2-mediated signaling, the JAK2/STAT3 signaling pathway is the critical target and biomarker during angiogenesis and tumor growth [15]. Moreover, the activated JAK2/STAT3 signaling pathway has been extensively validated as a novel molecular target for the treatment of human tumors by diverse drugs. Ponicidin induces cell cycle arrest in G0-G1 phase, apoptosis, ROS production and activated caspase-3 via JAK2/STAT3 signaling pathways in gastric carcinoma [15]. GL63 suppresses the proliferation of HCC cells by inhibition of the JAK2/STAT3 signaling pathway [16].

Because of the critical role of JAK2/STAT3 in HCC tumorigenesis, we investigated whether Op-B can mediate its effects in part through the suppression of the JAK2/STAT3 pathway. We found that Op-B indeed suppression of viability and invasion and induction of apoptosis, cell cycle arrest as well as JAK2/STAT3 signaling. In conclusion, our data indicate that inhibition of JAK2/STAT3 signaling pathway may correlate with Op-B-mediated suppression of MHCC97-H cell viability and invasion.

Materials and methods

Cell lines and cell culture

A human hepatocellular carcinoma cell line, MHCC97-H, was purchased from Cell Bank of Chinese Academy of Sciences. MHCC97-H cells were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone Laboratories, Inc., Logan Utah, USA), supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA), 100 mg/ml penicillin G and 50 μg/ml streptomycin (Life Technologies) maintained at 37°C with 5% CO_2 in a sterile incubator.

Cell viability assay

To detect cell viability following exposure to various concentrations (0, 5, 10 and 20 μM) of Op-B (Nanjing Ze Lang medical technology company, Nanjing, China), 100 μl MHCC97-H cells ($5×10^4$/ml) were seeded in 96-well plates and were cultured for 0, 12, 24, 48 and 72 h at 37°C. Viabilityof MHCC97-H cells was performed using CCK-8 kit purchased from Dojin Laboratories (Kumamoto, Japan). After treatment, 10 μl of Cell Counting kit (CCK)-8 reagents was added to each well and the cells were incubated at 37°C for another 1 h. The OD values were measured utilizing a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm wavelength.

Cell cycle analysis

MHCC97-H cells were seeded in 6-well plates at a density of $1×10^4$ cells/well and incubated at 37°C to allow cells to grow to 60%-80% confluence. Cells were treated with or without various concentrations (5, 10 and 20 μM) of Op-B for 48 h, and then harvested by centrifugation, washed with ice-cold phosphate-buffered saline (PBS), and fixed in ice-cold 70% ethanol overnight. The cells were then treated with 40 μg/ml RNase at 37°C and then stained with 40 μg/ml propidium iodide (PI) for 30 min. The percentage of cells in each phase (G0/G1, S, and G2/M) was calculated using a FACSC alibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis assay

Briefly, after MHCC97-H cells were seeded in 6-well plates at a density of $5×10^5$ cells/well and treated with or without various concentrations (5, 10 and 20 μM) of Op-B for 48 h, the cells were stained with annexin V and PI prior to analysis with the FACSCalibur flow cytometer. Cell death determinations included both early apoptotic cells (annexin V positive, PI negative)

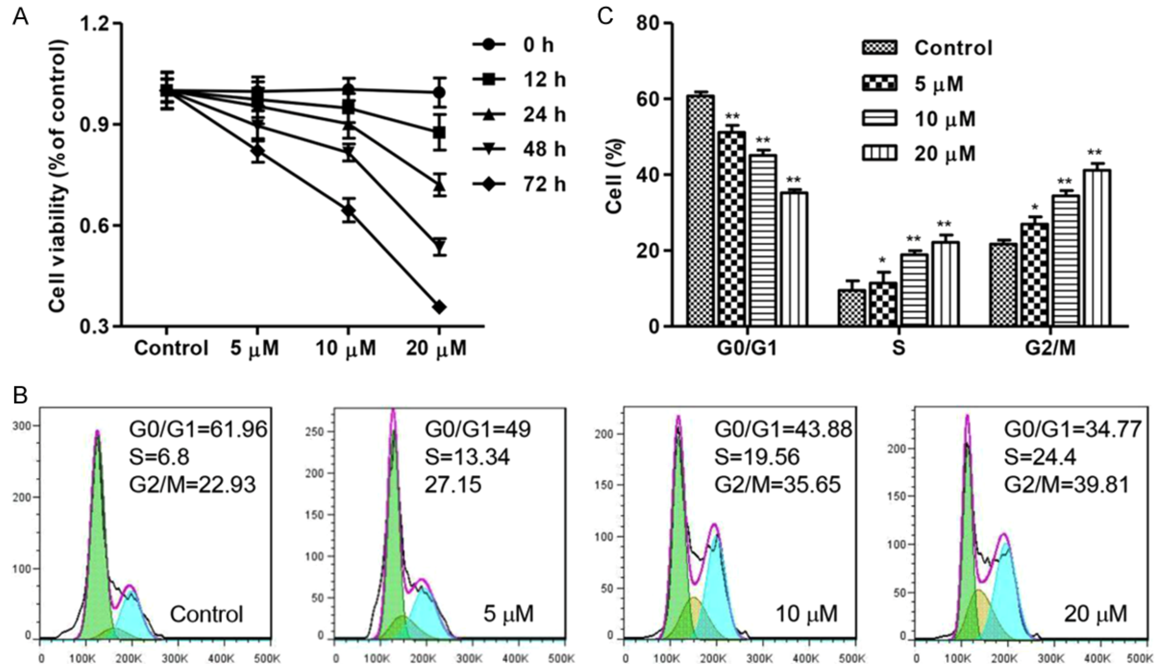

Figure 1. Op-B inhibits cell viability in MHCC97-H cells. MHCC97-H cells were treated with different concentrations of Op-B (5, 10 or 20 μM) for indicated time. A. Cell viability was determined by CCK-8 assay. B, C. Cell cycle was measured by flow cytometry assay. *P<0.05, **P<0.01 compared with control group.

and late apoptotic cells (annexin V positive, PI positive).

Matrigel invasion assay

The identification of MHCC97-H cell invasion was assayed using a 24-well Transwell chamber with a pore size of 8 μm. Cells were trypsinized after treated with or without various concentrations (5, 10 and 20 μM) of Op-B for 48 h and transferred to the upper Matrigel chamber in 100 μl of serum free medium containing 1×10^5 cells and cultured for 24 h. The lower chamber was filled with medium that contained 10% FBS as chemo attractants. After incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed and stained using 0.1% crystal violet. The numbers of invaded cells were counted in five randomly selected high power fields under a microscope.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by using 1 μg of total RNA, 0.5 mMdNTPs, 2.5 μM oligo-dT primer (for mature mRNAs), 1 U/μlRiboLock RNase Inhibitor (Thermo Fisher Scientific, Rockford, IL, USA) and 10 U/μl M-MuLVReverse Transcriptase (Thermo Fisher Scientific). The cDNA synthesis was performed for 1 h at 37°C and the reaction was stopped by 10 min incubation at 70°C. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific). Relative expression levels at the tested experimental conditions were calculated within each independent experiment using the using the ΔΔCt method. The sequences of the primer pairs were listed as follows: PCNA 5'-GGTGTTGGAGGCACTCAAGG-3' and 5'-CAGGGTGAGCTGCACCAAAG-3'; Bax 5'-AGCTGAGCGAGTGTCTCAAG-3' and 5'-TGTCCAGCCCATGATGGTTC-3'; Caspase-3 5'-AACTGGACTGTGGCATTGAG-3' and 5'-ACAAAGCGACTGGATGAACC-3'; Bcl-2 5'-CATGCACCAAGTCCAGTACAG-3' and 5'-TACAGGCATTGCCGCATAG-3'; MMP-2 5'-TTGACGGTAAGGACGGACTC-3' and 5'-GGCGTTCCCATACTTCACAC-3'; VEGF 5'-TCGAGACCCTGGTGGACATC-3' and 5'-CACACAGGACGGCTTGAAGA-3'; VEGFR-2 5'-GTGACCAACATGGAGTCGTG-3' and 5'-CCAGAGATTCCATGCCACTT-3'; GAPDH 5'-CACCCA-

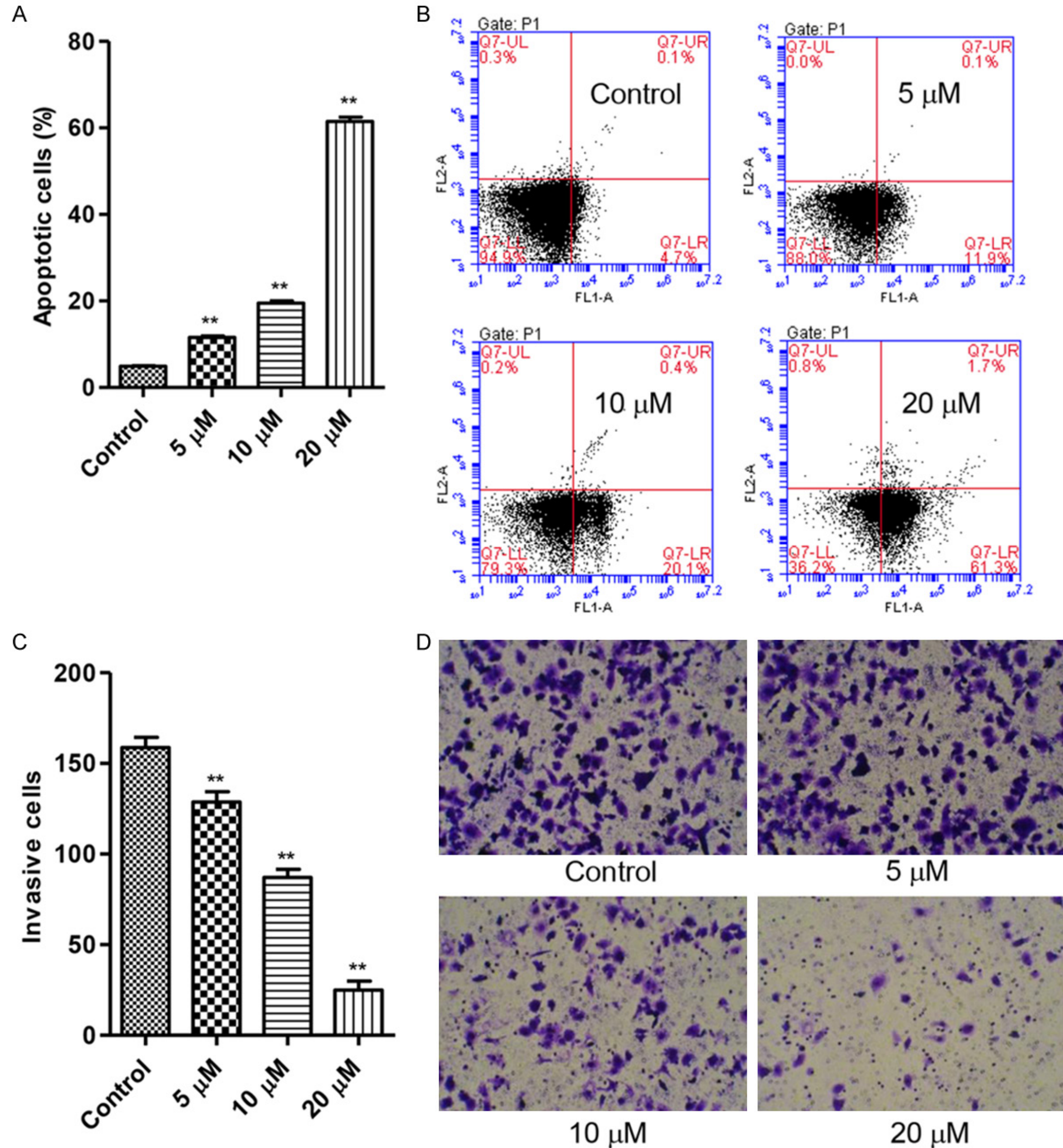

Figure 2. Op-B induces cell apoptosis and inhibits cell invasion in MHCC97-H cells. MHCC97-H cells were treated with different concentrations of Op-B (5, 10 or 20 μM) for 48 h. A, B. Cell apoptosis was determined by flow cytometry assay. C, D. Cell invasion was measured by Matrigel invasion assay. **$P<0.01$ compared with control group.

CTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTT-GCTGTAG-3'. Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantification, and all samples were normalized to GAPDH, which was used as an endogenous control.

Protein extraction and Western blotting

After treating with Op-B for 3 or 48 h, the cells were lysed in cold RIPA buffer (50 mM Tris, 5 mM EDTA, 1% Triton X-114, 0.4% sodium cacodylate, and 150 Mm NaCl), in the presence of protease inhibitors (1 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM β-glycerophosphate, 1mM sodium orthovanadate, and 1 mM PMSF). Then cells were homogenized and the protein concentrations were determined using a BCA kit. Samples were then boiled for 5 min, and 50 μg of protein was isolated with 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight with appropriate primary anti-

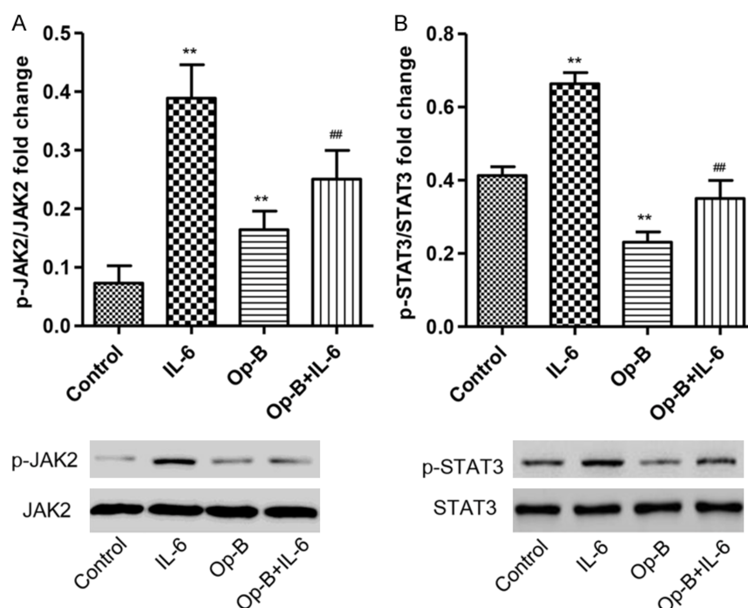

Figure 3. Op-B inhibits phosphorylation of JAK2 and STAT3 in MHCC97-H cells. MHCC97-H cells were treated with 10 μM of Op-B in the absence and presence of 10 ng/ml of IL-6 for 3 h. Expression levels of p-JAK2 (A), JAK2 (A), p-STAT3 (B) and STAT3 (B) were determined by Western blotting assay. **P<0.01 compared with control group. ##P<0.01 compared with Op-B group.

bodies against p-STAT3, STAT3, MMP-2, VEGF, VEGFR2 (Santa Cruz, CA, USA); p-JAK2, JAK2, PCNA, Bcl-2, Bax, Caspase-3 and GAPDH (Abcam, Cambridge, MA, USA). Blots were then incubated with horseradish peroxidase conjugated secondary antibody (Pierce, Rockford, IL, USA) and visualized by chemiluminescence.

Statistical analysis

Data are reported as means and SE for continuous variables. All cell culture experiments were performed at least three times and summarized. Statistical analysis of the data was performed using SPSS 16.0 software system (SPSS, Chicago, IL, USA). Comparison between groups was made using one-way ANOVA followed by Tukey's post hoc test. A *p*-value of less than 0.05 was considered significant.

Results

Op-B inhibits MHCC97-H cell viability and causes cell cycle arrest

The effects of Op-B on the cell viability of MHCC97-H were determined by the CCK-8 assay. Treating cellswith various concentrations of Op-B (5, 10 and 20 μM) for 0, 12, 24, 48 and 72 h resulted in significant decreases in viable cells ina time-dependent manner, and there were significant differences among all concentration groups (P<0.01; **Figure 1A**).

To determine whether Op-B affected cell cycle distribution, the flow cytometry assay was used. Treatment of MHCC97-H cells with various concentrations of Op-B (5, 10 or 20 μM) for 48 h significantly decreased the percentages of cells in the G0/G1 phase compared with the control, whereas increases in the percentages of cells in the S and G2/M phases (P<0.01; **Figure 1B**). The statistical results are shown in **Figure 1C** and the data are concentration-dependent.

Op-B induces apoptosis and inhibits invasion in MHCC97-H cells

Typical histograms of apoptosis in MHCC97-H cells treated with various concentrations of Op-B (5, 10 or 20 μM) for 48 h are shown in **Figure 2A**, **2B**. Exposure of cells to 5, 10 or 20 μM of Op-B resulted in significant increase in cell apoptosis by 1.38-, 3.00- and 11.64-fold compared with control cells, respectively (P<0.01). These findings demonstrate that Op-B can induce cell apoptosis.

To determine whether Op-B affected cell invasion, the Matrigel invasion assay was used. Treatment of MHCC97-H cells with various concentrations of Op-B (5, 10 or 20 μM) for 48 h significantly decreased the invasive cells by 18.9%, 45.2% and 84.4% compared with control cells, respectively (P<0.01; **Figure 2C**, **2D**). These findings demonstrate that Op-B can inhibit cell invasion.

Op-B inhibits phosphorylation of JAK2 and STAT3 in MHCC97-H cells

The JAK2/STAT3 signaling pathway, which is often associated with tumorigenesis and activated in numerous tumors, is well-known to

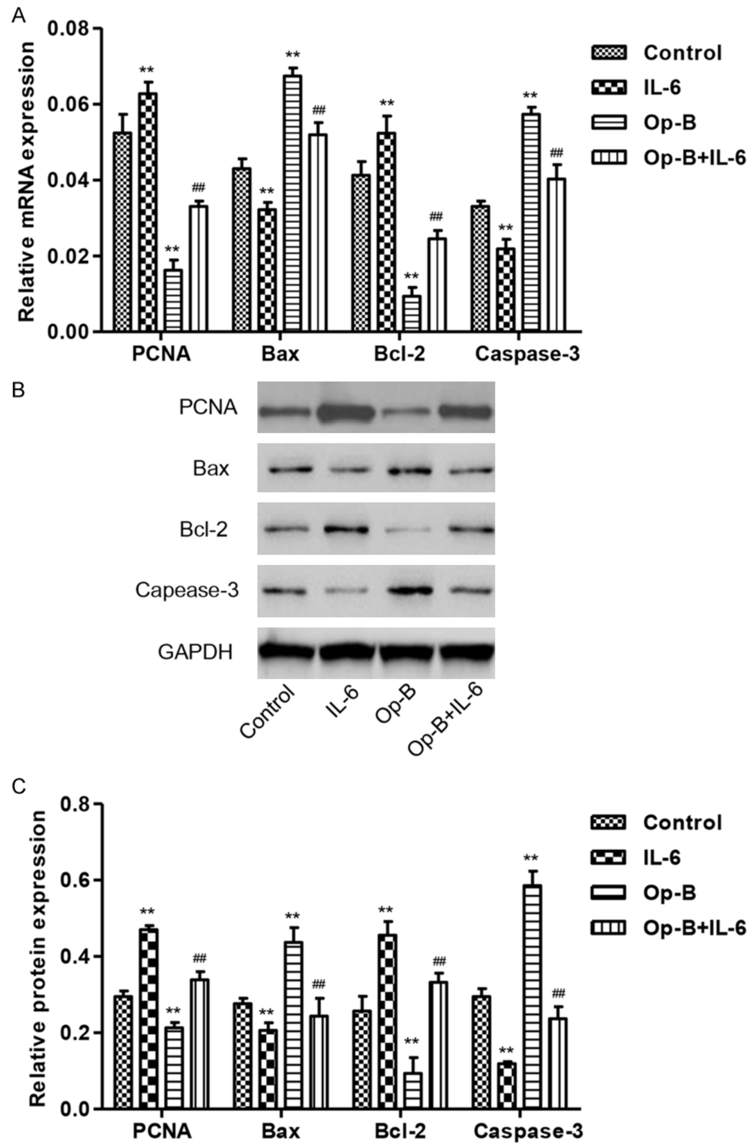

Figure 4. Effects of Op-B on regulators of cell apoptosis in MHCC97-H cells. MHCC97-H cells were treated with 10 μM of Op-B in the absence and presence of 10 ng/ml of IL-6 for 48 h. Expression levels of PCNA, Bax, Bcl-2 and Casapse-3 were determined by RT-qPCR (A) and Western blotting assay (B, C). **P<0.01 compared with control group. ##P<0.01 compared with Op-B group.

regulate apoptosis and invasion [15, 16]. Thus, the pathway was examined in relation to Op-B-induced apoptosis and invasion in MHCC97-H cells. 10 ng/ml of IL-6 was added into the MHCC97-H cells, which could induce STAT3 activation in many studies [17, 18]. When cells were treated with IL-6 for 3 h, p-JAK2 and p-STAT3 were significantly increasedin MHCC97-H cells (P<0.01), but JAK2 and STAT3 was not affected (**Figure 3A**, **3B**). Next, we tested the effect of Op-B on the JAK2/STAT3 pathway in MHCC97-H cells. As shown in **Figure 3**, contrary to IL-6, 10 μM of Op-B inhibited p-JAK2 and p-STAT3 (P<0.01). In contrast, JAK2 and STAT3 were not affected by any of the above treatments.

Down-regulation of PCNA and up-regulation of caspase-3 and Bax/Bcl-2 ratio may contribute to Op-B-mediated cell apoptosis

To explore the molecular mechanism of Op-B-mediated cell apoptosis, we quantified the expression of cell apoptosis-regulated proteins. RT-qPCR analysis revealed that treating MHCC97-H cells with 10 μM of Op-B for 48 h resulted in significant decreases in the levels of PCNA and Bcl-2 (P<0.01; **Figure 4A**). Whereas, the expression levels of other important regulators of cell apoptosis, such as Bax and Caspase-3, were significantly increased in response to Op-B treatment (P<0.01). However, IL-6 treatment significantly inhibited Op-B-induced increases in the expression of Bax and Caspase-3 and decreases in the expression of PCNA and Bcl-2 in MHCC97-H cells (P<0.01).Similar results were also found in MHCC97-H cells by Western blot analysis (**Figure 4B**, **4C**).

Down-regulation of MMP-2, VEGF and VEGFR2 may contribute to Op-B-mediated cell invasion reduction

To explore the molecular mechanism of Op-B-mediated cell invasion reduction, we quantified the expression of cell invasion-regulated proteins. RT-qPCR analysis revealed that treating MHCC97-H cells with 10 μM of Op-B for 48 h resulted in significant decreases in the levels of MMP-2, VEGF and VEGFR2 (P<0.01; **Figure 5A**). However, IL-6 treatment significantly in-

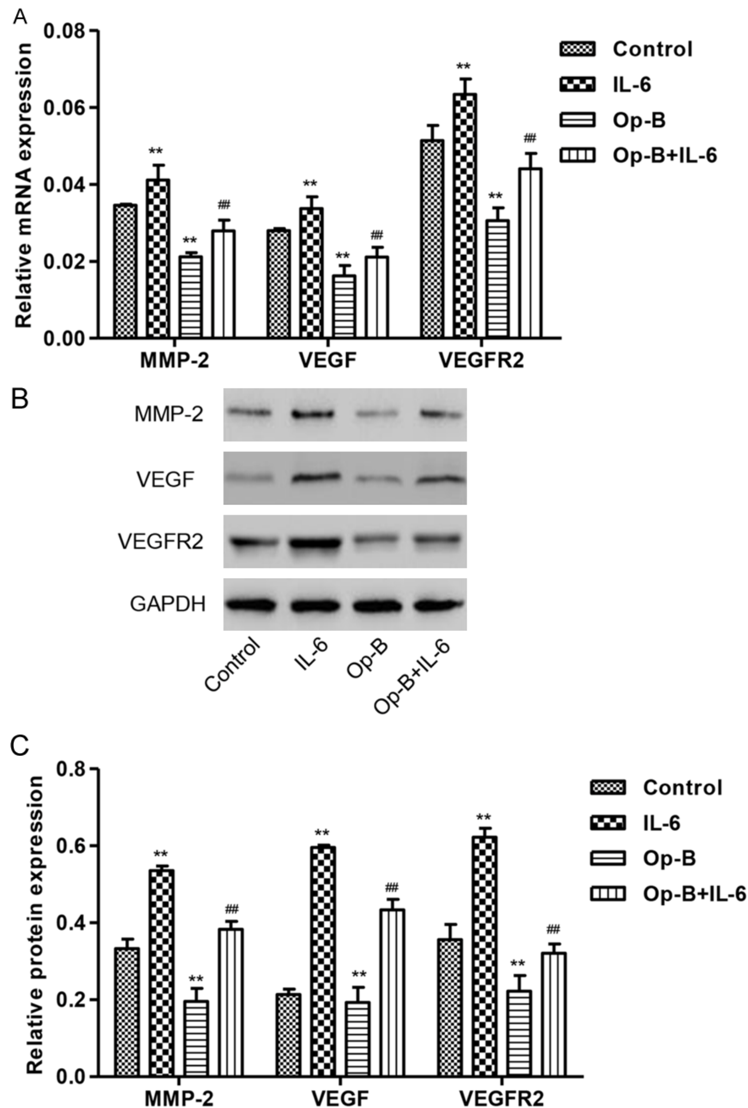

Figure 5. Effects of Op-B on regulators of cell invasion in MHCC97-H cells. MHCC97-H cells were treated with 10 μM of Op-B in the absence and presence of 10 ng/ml of IL-6 for 48 h. Expression levels of MMP-2, VEGF and VEGFR2 were determined by RT-qPCR (A) and Western blotting assay (B, C). **P<0.01 compared with control group. ##P<0.01 compared with Op-B group.

hibited Op-B-induced decreases in the expression of MMP-2, VEGF and VEGFR2 in MHCC97-H cells (P<0.01). Similar results were also found in MHCC97-H cells by Western blot analysis (**Figure 5B**, **5C**).

Discussion

Although curative surgery and chemotherapy offer an opportunity for HCC patients, the cumulative 5-year recurrence rate still ranges from 40% to 80% because of high potential for vascular invasion and metastasis [19, 20]. Consequently, the identification of medicine encompasses a vital finding and an opportunity to improve HCC treatment. The present study confirmed that high concentrations of Op-B were able to significantly inhibit the viability and invasion of MHCC97-H cells, and induce cell cycle arrest in S and G2/M phases and apoptosis in a dose-dependent manner. To determine whether Op-B exerts its anticancer effects in HCC cells through the abrogation of the JAK2/STAT3 signaling pathway, IL-6 was also introduced. We found that Op-B suppressed JAK2/STAT3 activation and also down-regulated the expressions of STAT3-regulated gene products, including PCNA, Bcl-2, MMP-2, VEGF and VEGFR2, which was corrected by IL-6 treatment.

Op-B is a natural active compound extracted from the Chinese herbal medicine Radix*O. japonicas*. In the present study, our results showed that Op-B significantly induced MHCC97-H cellcycle arrest in S and G2/M phases as well as apoptosis through decreasing the expression of PCNA and Bcl-2 and increasing expression of Caspase-3 and Bax. Consistent with our findings, previous study showed that Op-B induced adenocarcinoma A549 cell death and that, was caspase and mitochondrial independent, cell cycle arrest in the S and G2/M phases by inhibiting the expression of Myt1 and phosphorylation of Histone H3 (Ser10) [21]. PCNA, as an index of the state of cell proliferation and indication of the malignant degree, was significantly enhanced in the nucleus of hepatocarcinoma cells, which has been confirmed in numerous studies [22, 23]. In the present study, our

results showed that Op-B significantly inhibited MHCC97-H cell invasion through decreasing the expression of MMP-2, VEGF and VEGFR2. Consistent with our findings, Op-B inhibits cell adhesion, invasion and migration in non-small cell lung cancer (NSCLC) A549 cells through down-regulation of MMP-2/9 and p-Akt [24]. MMPs are a family of related zinc-dependent proteinases that are believed to play important role in the invasive process and metastasis of cancer cells [25]. Among them, MMP-2 and MMP-9 are the most concerned and their functions have been well-characterized in HCC [26]. VEGF appears to serve at least two key roles in tumor progression. In addition to its known angiogenic action, VEGF also appears to promote tumor-initiating cell expansion and enhance the robustness of metastatic progenitors [14]. VEGFR2 is a tyrosine kinase receptor essential for VEGF mediated physiological and pathological responses in endothelial cells [27]. Although initially thought to be exclusively expressed by endothelial cells, VEGFR2 is also expressed by different cancers and VEGF/VEGFR2 can act via both autocrine and paracrine mechanisms to drive cancer cell proliferation and survival [28].

The present study further explored the role of the JAK2/STAT3 signaling pathway in the anti-hepatocarcinoma effect of Op-B for the first time and evaluated that Op-B could efficiently inhibit the phosphorylation of JAK2 and STAT3, thus affecting cell viability and inducing apoptosis. The effects of Op-B on STAT3 phosphorylation correlated with the suppression of upstream JAK2. Compared with Op-B treatment, IL-6 stimulation decreased the efficacy of induction of apoptosis of MHCC97-H cells. IL-6 treatment resulted in activation in JAK2 and STAT3 phosphorylation and expression of PCNA and Bcl-2, inactivated caspase-3 and Bax, and inhibited apoptotic death of cells remarkably. This finding verifies previous studies reporting that the JAK2/STAT3 signaling pathway is associated with the apoptosis by the intrinsic mitochondrial pathway in hepatocarcinoma cells [29]. Previous study provides evidence that VEGF-bound VEGFR2 recruits JAK2 and STAT3 to stimulate STAT3-dependent induction of embryonic stem cell transcription factors [14]. Activation of VEGFR2-mediated JAK2/STAT3 contributes to the gastric carcinoma tumorigenesis [15]. All of these results clearly demonstrated that the apoptosis and decreased invasion induced by Op-B partially via JAK2/STAT3 signaling pathway is an important mechanism underlying HCC development and metastasis.

The pharmacological assays conducted in the present study suggested that Op-B significantly decreased viability and caused cell cycle arrest in MHCC97-H cells in a dose-dependent manner. Op-B also inhibited invasion and induced apoptosis of MHCC97-H cells. Mechanistically, it was through inhibiting JAK2/STST3 signaling pathway. Thus, we speculate that Op-B may be an alternative agent in the treatment of HCC. The further studies on the underlying mechanism of OP-B and the doses of OP-B that effectively demonstrate preventive effects without toxicity for humans are undergoing and will be reported in due course.

Disclosure of conflict of interest

None.

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