# IL-6 plays a crucial role in epithelial-mesenchymal transition and pro-metastasis induced by sorafenib in liver cancer

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**Abstract.** Interleukin-6 (IL-6) is involved in various biological responses, including tumor progression, metastasis and chemoresistance. However, the role and molecular mechanism of IL-6 in the treatment of sorafenib in liver cancer remain unclear. In the present study, through western blot analysis, Transwell assay, flow cytometric assay, ELISA analysis and immunohistochemistry it was revealed that sorafenib promoted metastasis and induced epithelial-mesenchymal transition (EMT) in liver cancer cells *in vitro* and *in vivo*, and significantly increased IL-6 expression. Endogenous or exogenous IL-6 affected metastasis and EMT progression in liver cancer cells through Janus kinase 2/signal transducer and activator of transcription 3 (STAT3) signaling. Knocked out IL-6 markedly attenuated the pro-metastasis effect of sorafenib and increased the susceptibility of liver cancer cells to it. In conclusion, the present results indicated that IL-6/STAT3 signaling may be a novel therapeutic strategy for liver cancer.

## Introduction

Liver cancer is the sixth most common type of cancer and the third leading cause of cancer mortality worldwide (1). Although surgical resection is the primary and most effective treatment for patients with liver cancer, prognosis following surgical resection remains poor, due to the high 5-year recurrence rate (~70%) (2,3). However, most patients with liver cancer are diagnosed with advanced disease, consequently missing the window of opportunity for surgery, with the overall 5-year survival rate of patients with liver cancer at only 17% (1,4). Sorafenib, an orally active multi-targeted tyrosine kinase inhibitor, is the first FDA-approved molecular targeted therapy available for advanced liver cancer, and has been used as first-line therapy (5). Nevertheless, the therapeutic effect of sorafenib is limited, and the median survival time of patients is increased by ~3 months (6,7). Moreover, several side effects of sorafenib have been reported. Patients who discontinue sorafenib due to chemoresistance or severe side effects may suffer tumor recurrence, with rapid tumor progression after they stop taking the drug (8). Sorafenib can also promote liver cancer cell invasion and migration, as demonstrated by western blot analysis and in an *in vivo* tumor model (9). Therefore, the molecular mechanism of sorafenib should be fully understood, and novel therapeutic strategies for liver cancer should be explored.

Interleukin-6 (IL-6) is a pleiotropic cytokine present in the tumor microenvironment and involved in various biological responses, including tumor progression, metastasis and chemoresistance (10). IL-6 plays a crucial role in linking chronic inflammation to liver cancer progression (11-13), and the expression of the IL-6 gene is associated with tumor stage in liver cancer (14). IL-6 levels in cancer tissues and serum were increased in patients with liver cancer, as compared with healthy controls; IL-6 levels were also correlated with tumor metastasis and reduced patient survival (15,16).

Epithelial-mesenchymal transition (EMT) is a biological process involved in various physiological and pathological processes and tumorigenesis (17,18). During EMT, tumor cells lose their epithelial traits, such as cell polarity and cell-cell adhesion, and gain mesenchymal characteristics, such as migration, invasion and anti-apoptosis (19). EMT is involved in invasive, metastatic and therapeutic resistance in liver cancer (20-23). IL-6 is likely a potent triggering factor in the mediation of EMT in various types of cancer, such as breast, head and neck, and colon cancer (24-26).

The aim of the present study was to further explore the molecular mechanism of the sorafenib-mediated pro-metastatic effect and resistance in liver cancer, and the role of IL-6 in sorafenib treatment.

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## Materials and methods

*Cell culture and drugs*. Hepatocellular carcinoma (HCC)LM3 and HepG2 cells (both obtained from the Liver Cancer Institute, Fudan University, Shanghai, China), and authenticated by STR profiling, were cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences; Cytiva) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 50 mg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ at 37˚C. Sorafenib (Bayer AG) was prepared as previously described (9). Recombinant human IL-6 (cat. no. 206-IL-010) was purchased from R&D Systems (R&D Systems, Inc.). The JAK inhibitor AG490 was procured from Selleck Chemicals.

*Stable cell line construction by transcription activator-like effector nucleases (TALEN)*. Stable cell lines were constructed in accordance with previously described procedures (27). First, the TALEN design was in accordance with the sequence of IL-6. The TALEN arms were designed as 2x3 (2 left and 3 right arms) combination targets on the IL-6 (NCBI gene ID, 3569). The plasmids for the left and right arms of the TALEN were constructed using the FAST TALEN Kit according to the manufacturer's instructions (SIDANSAI Biotechnology). Following sequencing, 5 plasmids were transfected into the 293T cell line (obtained from the Chinese Academy of Sciences) for 24 h at 37˚C using FuGene HD transfection reagent (Roche Diagnostics) in a 2x3 cross combination. A pair of TALEN plasmids was selected as the most effective knockout group after 3 days of puromycin screening and subsequent genomic PCR sequencing. The HCCLM3 cell line was routinely culture-dand was plated for 16 h before transfection. The HCCLM3 cell line was transfected with the indicated plasmids for 24 h at 37˚C using Fugene HD (Roche Diagnostics), according to the manufacturer's instructions. The selected pair of TALEN plasmids, which had the highest cleavage efficiency, was co-loaded into the HCCLM3 cell line. The amount of plasmids per well for the 6-well plates included in each transfection were 2 $\mu$g pTALEN-Left, 2 $\mu$g pTALEN-Right, and 0.5 $\mu$g of pEGFP as a transfection marker. The cells were exposed to 2 $\mu$g/ml puromycin for 3 days. The medium containing puromycin was then replaced with growth media. After a week of monoclonal culturing, stably transfected clones [HCCLM3-IL-6(-)] were validated through western blot analysis and reverse transcription quantitative polymerase chain reaction (RT-qPCR), as compared with HCCLM3-wild-type (wt), which was not transfected with TALEN plasmids. The following primers of IL-6 were used: 5'-GAACTCCTTCTCCACAAGCG-3' forward and 5'-TTT TCTGCCAGTGCCTCTTT-3' reverse.

*Cell proliferation assay and flow cytometry*. The cell proliferation assay was performed using a Cell Counting Kit-8 (CCK-8) assay kit according to the manufacturer's instructions. In this procedure, $4x10^3$ cells were seeded in each well of a 96-well plate and cultured for different time-points (0, 24 and 48 h). The cells were then incubated with 100 $\mu$l DMEM containing 10% CCK-8 reagent (Dojindo Molecular Technologies, Inc.) in each well at 37˚C for 2 h, and absorbance was detected at a wavelength of 450 nm using a microplate reader (Nexcelom, Inc.).

The reagents of apoptosis and the cell cycle were used in accordance with the manufacturer's protocol before flow cytometry was performed. For the apoptosis assay, single-cell suspensions were prepared, and then $1x10^5$ cells were washed with phosphate-buffered saline (PBS) twice and stained with Annexin V and propidium iodide (BD Biosciences). For the cell cycle assay, $1x10^5$ cells were incubated in 75% ethanol at -20˚C overnight and stained with PI/RNase staining buffer (BD Biosciences) at room temperature for 15 min. Fluorescence was measured using FACSCalibur (BD Biosciences) and analyzed using FlowJo v7.6.1 software (Tree Star, Inc.).

*Western blot analysis*. Western blot analysis was performed in accordance with previously described procedures (28). The brief steps were as follows: 30 $\mu$g Protein extracted from the cells was subjected to 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The extract was then blocked with 5% defatted milk for 1 h at room temperature. The membrane was incubated with a primary antibody at 4˚C overnight. On the following day, the membrane was incubated with HRP-conjugated anti-mouse/rabbit secondary antibody at a dilution of 1:5,000 with 5% defatted milk for 1 h at room temperature. The bands were detected using a ChemiDoc MP system (Bio-Rad Laboratories, Inc.).

The following primary antibodies were used: IL-6 (1:2,000; cat. no. NB600-1131; Novus Biologicals), E-cadherin (1:1,000; product no. 3195T), N-cadherin (1:1,000; product no. 13116T), Vimentin (1:1,000; product no. 5741T), Snail (1:1,000; product no. 3879T), Janus kinase 2 (JAK2) (1:1,000; product no. 3230T), phospho (p)-JAK2 (1:1,000; product no. 3771S), STAT3 (1:1,000; product no. 9139T), p-STAT3 (1:1,000; product no. 9145T), cyclin dependent kinase 2 (CDK2) (1:1,000; product no. 18048T), cyclin D1 (1:1,000; product no. 55506T), cleaved caspase-3 (1:1,000; product no. 9661T), cleaved poly (ADP-ribose) polymerase (PARP) (1:1,000; product no. 5625T), B-cell lymphoma-2 (Bcl-2) (1:1,000; product no. 15071T) and β-actin (1:1,000; product no. 4970T; all from Cell Signaling Technology, Inc.). A peroxidase-conjugated goat anti-rabbit/mouse secondary antibody was purchased from YEASEN Biotech (1:5,000; cat. nos. 33101ES60/33201ES60; YEASEN Biotech, Inc.).

*RT-qPCR*. RNA isolation and RT-qPCR procedures were performed as previously described (27). TB Green Premix Ex Taq II (cat. no. RR820A) and PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (cat. no. RR047A) were purchased from Takara Bio, Inc., and primers were synthesized by Sangon Biotech. The primers for IL-6 were used as described above and the primers for GAPDH were used as follows: Forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

*ELISA*. The cell supernatant protein levels of IL-6 were analyzed by ELISA using IL-6 Quantikine® ELISA kit according to the manufacturer's instructions (cat. no. D6050; R&D Systems, Inc.). Equal numbers ($1x10^6$) of HCCLME3-wt cells and HepG2-wt cells were plated and cultured for 24 h. The cells were then incubated with or without sorafenib (5 or

10 $\mu$mol/l) at 37˚C for 24 h. ELISA was performed using the cell supernatant collected. All analyses were performed in triplicate.

*Cell migration assay*. Cell migration was assessed by Transwell assay in Boyden chambers with an 8-$\mu$m pore (Corning Inc.). The cells were incubated with or without sorafenib (5 or 10 $\mu$mol/l) or IL-6 (50 ng/ml) at 37˚C for 24 h. Next, $5x10^4$ cells in 200 $\mu$l serum-free DMEM were seeded onto the upper chamber, and 650 $\mu$l DMEM containing 10% BSA was perfused to each well in the lower chamber. After the non-migrating cells were removed, the remaining cells were fixed with 100% methanol for 15 min at room temperature, stained with 0.1% crystal violet dye for 5 min at room temperature, and finally counted under a light microscope at a magnification of x100. Three independent experiments were performed in triplicate.

*Xenograft model of human liver cancer in nude mice*. To form subcutaneous tumors, HCCLM3-wt and HCCLM3-IL-6(-) cells ($1x10^7$ cells) were mixed with PBS and injected into the right flank of four, 4-week-old male BALB/c nude mice (2 mice per group) (Beijing Vital River Laboratory Animal Technology) weighing approximately 20 g which were housed in an appropriate environment (28˚C; ~40-60% humidity; 10-h light/14-h dark cycle; plenty of sterilized food and water, laminar flow cabinet under specific pathogen-free conditions). After 4 weeks, the mice were sacrificed by cervical vertebra dislocation, and the tumor tissues were cut into 1-$cm^3$ pieces and implanted into the livers of the nude mice (a total 24 of mice were used; 12 mice per group) anesthetized using intraperitoneal anesthesia with pentobarbital sodium (2.5 mg/kg) as previously described (29). The treatment started 1 week after the tumor was orthotopically implanted. Each group of mice was divided into two subsets containing 6 mice and treated with 30 mg/kg/day sorafenib or vehicle for 5 weeks. Following mouse sacrifice by cervical vertebra dislocation, the lung tissues were extracted and analyzed after hematoxylin and eosin (H&E) staining at room temperature for 5 min. Lung metastases were examined as previously described (29). Ten slices from each lung were observed. The animal experiments were approved by the Animal Care Committee of Zhongshan Hospital (Shanghai, China).

*Immunohistochemistry*. Immunohistochemical staining was performed as previously described (29). Paraffin-embedded orthotopically implanted tumors were cut into 5-$\mu$m sections and then deparaffinized and rehydrated. Immunohistochemical staining was performed using the Ultra Vision Quanto Detection HRP DAB System (cat. no. TL-015-QHD; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Briefly, the slices from orthotopically implanted tumors were treated with a diluted primary antibody against IL-6 (1:100; cat. no. NB600-1131; Novus Biologicals) at 4˚C overnight and anti-rabbit/mouse secondary antibodies (included in the Ultra Vision Quanto Detection HRP DAB System) at room temperature for 60 min. Signals were detected by DAB at room temperature for 5 min. Immunohistochemical images were recorded using a computerized image system composed of a Leica CCD camera DFC420 connected to a Leica DM IRE2 microscope (Leica Microsystems, Inc.). The total positive staining area of IL-6 was calculated by Image-Pro Plus v6.2 software (Media Cybernetics, Inc.).

*Statistical analysis*. Data were analyzed with SPSS 18.0 (SPSS Inc.). Quantitative variables were analyzed by unpaired two-tailed Student's t-test or one-way ANOVA with Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*IL-6 knockout attenuates the pro-invasive effect induced by sorafenib treatment in vitro and in vivo*. In Fig. 1A and B, sorafenib significantly increased the number of metastatic cells in HCCLM3-wt and HepG2-wt cells after treatment for 24 h. After IL-6 expression was disrupted by TALEN, which is a highly efficient and specific gene editing tool with low genotoxicity in targeted genome manipulation (30,31), in a human liver cancer HCCLM3-wt cell line, the stably transfected clones were validated by RT-qPCR and western blot analysis (Fig. 1C and D). Notably, sorafenib did not increase the number of metastatic cells in HCCLM3-IL-6(-) (Fig. 1E).

Subsequently, the orthotopic growth of liver cancer tumors was modeled in nude mice to further investigate whether IL-6 influenced the pro-migratory effect of sorafenib *in vivo*. It was revealed that sorafenib significantly reduced the volume of tumors in the HCCLM3-wt and HCCLM3-IL-6(-) groups, respectively (Fig. 2A and B). However, intrahepatic metastasis (IHM) was increased in the HCCLM3-wt group but not in the HCCLM3-IL-6(-) group following the administration of sorafenib (Fig. 2C and D). The number of IHMs in the HCCLM3-wt control was not higher than that in the HCCLM3-IL-6(-) control (Fig. 2C and D). Moreover, sorafenib treatment significantly increased the number of lung metastatic nodules in HCCLM3-wt cells (Fig. 2E and F). Conversely, no significant difference was observed in the two groups of mouse HCCLM3-IL-6(-) cells (Fig. 2E and F). The number of lung metastases in the HCCLM3-wt group was higher than that in the HCCLM3-IL-6(-) group (Fig. 2E and F). These results indicated that IL-6 knockout attenuated the pro-migratory effect induced by sorafenib treatment, as detected by western blot analysis and *in vivo*.

*Sorafenib may promote liver cancer cell metastasis and EMT through the upregulation of IL-6, as detected by western blot analysis, ELISA analysis and immunohistochemistry*. To explore the role of IL-6 in sorafenib-mediated pro-metastasis and EMT, HCCLM3-wt and HepG2-wt cells were treated with sorafenib. As revealed in Fig. 1A and B, sorafenib significantly promoted metastasis in HCCLM3-wt and HepG2-wt cells at 24 h. Furthermore, western blot analysis indicated that 0-10 $\mu$mol/l sorafenib induced EMT in HCCLM3-wt and HepG2-wt cells at 24 h (Fig. 3A and B). It was also determined that IL-6 was upregulated after 24 h of treatment with sorafenib in HCCLM3-wt and HepG2-wt cells (Fig. 3C and D). In addition, ELISA and immunohistochemistry were performed to detect the change of IL-6 in the cell supernatant and inside the tumor following sorafenib administration. Consistent with the western blot analysis results, it was revealed that IL-6

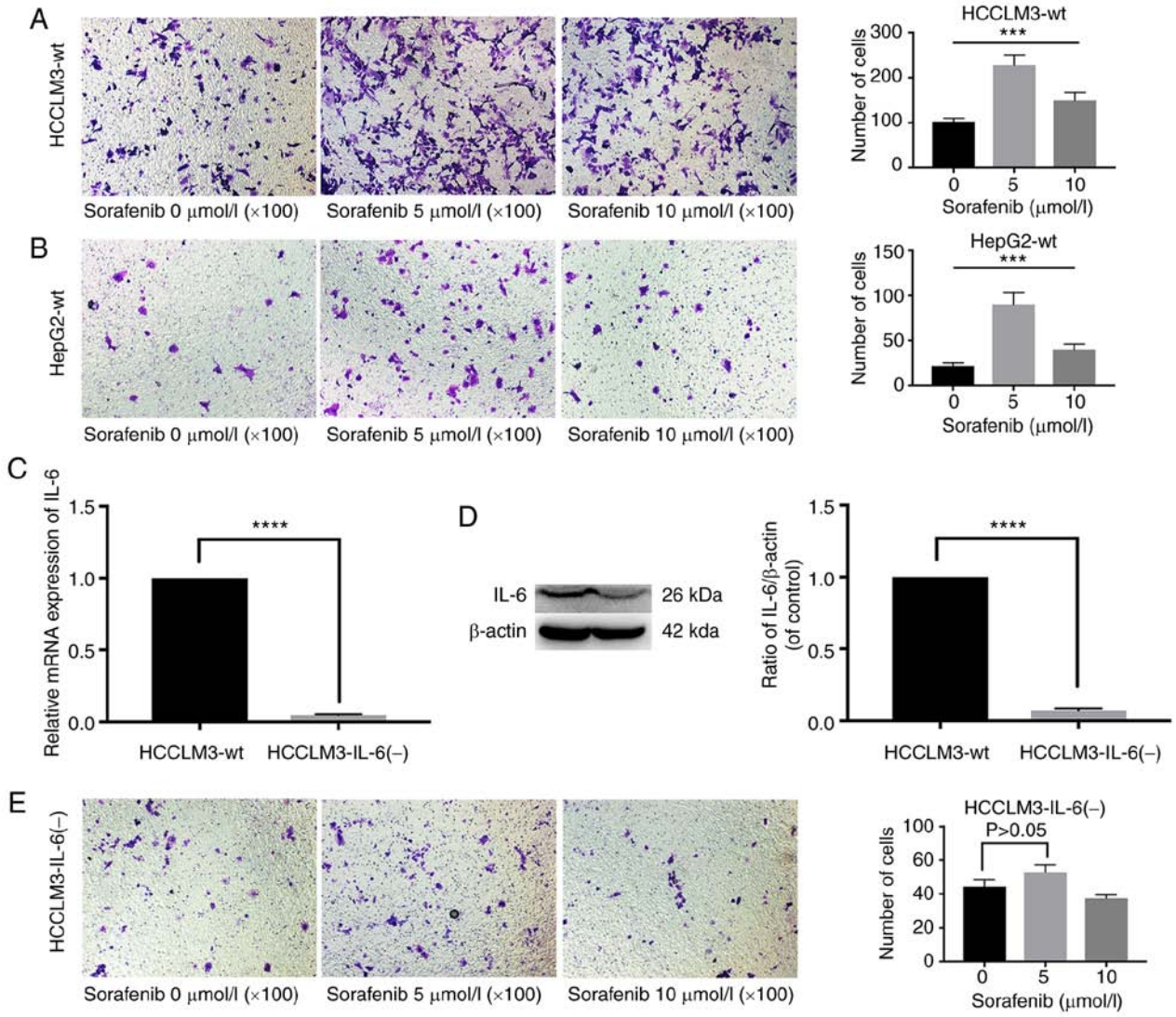


Figure 1. Sorafenib increases the metastatic potential of liver cancer cells and IL-6 knockout attenuates the pro-invasive effect induced by the treatment of sorafenib. (A and B) Transwell assays revealed that sorafenib increased the metastatic potential of HCCLM3-wt and HepG2-wt cells (both $^{***}P<0.001$). (C and D) IL-6 expression was disrupted by TALEN. Stably transfected clones were validated through RT-qPCR and western blot analysis (both $^{****}P<0.0001$). (E) Transwell assays revealed that IL-6 knockout attenuated the pro-invasive effect induced by sorafenib in HCCLM3-IL-6(-) ($P>0.05$). HCC, hepatocellular carcinoma; IL-6, interleukin-6; wt, wild-type; TALEN, transcription activator-like effector nucleases; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

was upregulated in the cell supernatant and inside the tumor *in vivo* (Fig. S1). These results indicated that the pro-metastatic effects of sorafenib may be exerted through the upregulation of IL-6 expression in liver cancer cells.

*IL-6 knockout inhibits the proliferation of HCCLM3 cells and promotes apoptosis in HCCLM3 cells, as detected by CCK-8 assay, flow cytometric analysis and western blot analysis*. The influence of the disruption of the IL-6 expression was examined by TALEN in HCCLM3 cells. Western blot analysis and RT-qPCR were performed to confirm the stable knockout of IL-6 expression in HCCLM3 cells (Fig. 1C and D). A CCK-8 assay indicated that IL-6 promoted tumor cell proliferation (Fig. 4A). To further explore the effects of IL-6 expression on liver cancer growth, flow cytometry was conducted to detect the cell cycle and apoptosis of HCCLM3 cells. In Fig. 4B and C, HCCLM3-IL-6(-) increased the G1 phase and apoptosis rates, as compared with those of HCCLM3-wt cells. In addition, the level of cell cycle (CDK2 and cyclin D1) and apoptotic (cleaved caspase-3, cleaved PARP and Bcl-2) markers was investigated by western blot analysis. The results revealed that Bcl-2, cyclin D1 and CDK2 levels were higher in HCCLM3-wt cells than in HCCLM3-IL-6(-) cells, whereas those of cleaved caspase-3 and cleaved PARP were upregulated in HCCLM3-IL-6(-) cells (Fig. 4D). These results revealed that IL-6 knockout inhibited the proliferation and promoted the apoptosis of HCCLM3 cells.

*IL-6 knockout decreases the metastatic ability of HCCLM3 cells, and exogenous IL-6 increases that of HepG2 and HCCLM3 cells, as detected by Transwell assay and western blot analysis*. The influence of IL-6 disruption on the metastatic ability of HCCLM3 cells was explored. A Transwell assay was performed to evaluate the metastatic ability of HCCLM3 cells. When IL-6 was knocked out, the migration of HCCLM3-IL-6(-) cells significantly decreased, as compared with that of HCCLM3-wt cells (Fig. 5A). Consistent with the Transwell assay results, western blot

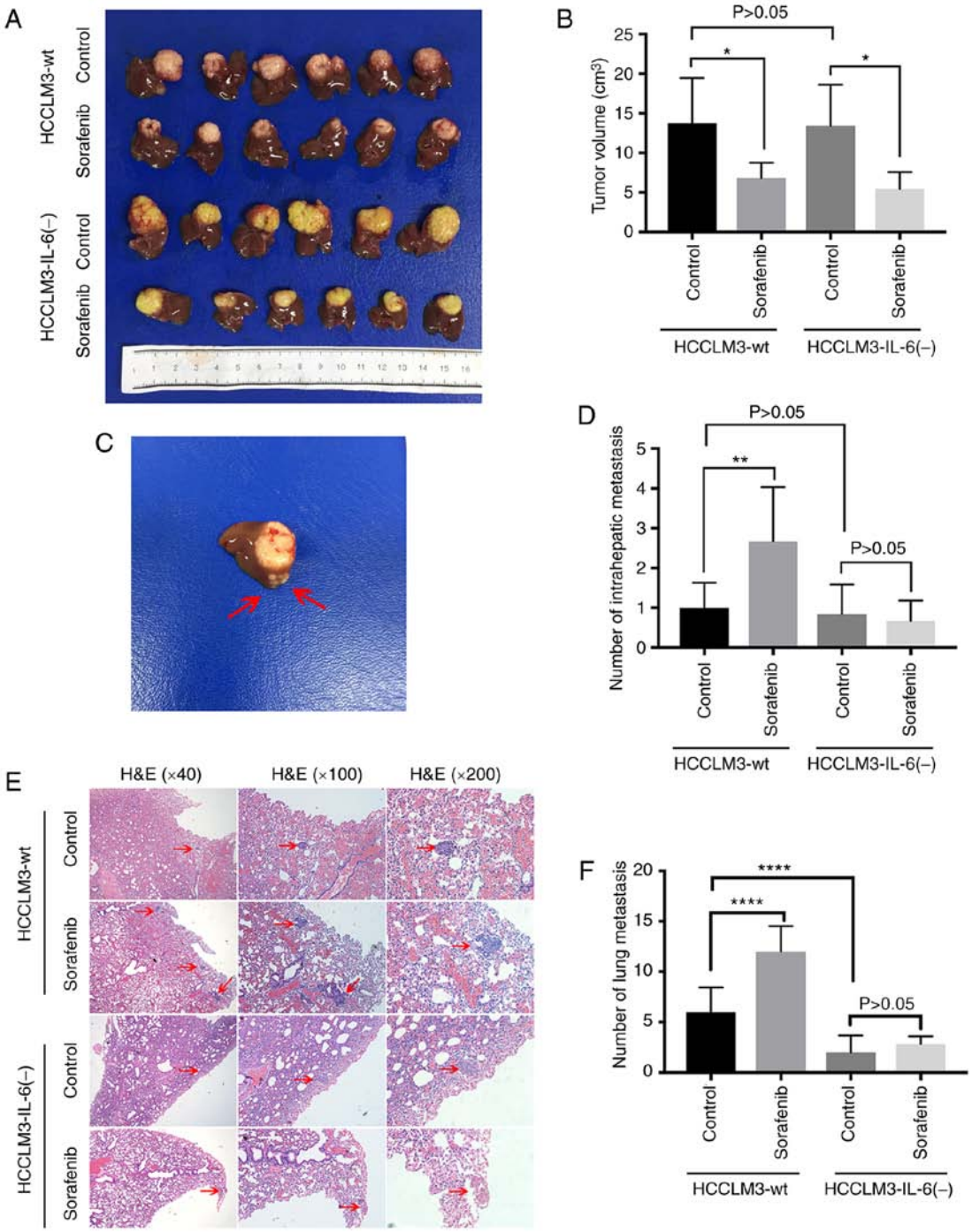


Figure 2. Sorafenib decreases the tumor volume and increases the intrahepatic metastatic potential and lung metastatic potential of liver cancer cells *in vivo*. IL-6 knockout attenuated the pro-invasive effect induced by the treatment of sorafenib *in vivo*. (A and B) Sorafenib decreased the tumor volume in the HCCLM3-wt and HCCLM3-IL-6(-) groups (both $^{*}$P<0.05). (C and D) Sorafenib increased the number of IHMs in the HCCLM3-wt sorafenib group compared with the HCCLM3-wt control, while sorafenib did not increase the number of IHMs in the HCCLM3-IL-6(-) sorafenib group compared with the HCCLM3-IL-6(-) control. ($^{**}$P<0.01 and P>0.05, respectively). The number of IHMs in the HCCLM3-wt control was not higher than that in the HCCLM3-IL-6(-) control (P>0.05). (E and F) Sorafenib increased the number of lung metastases in the HCCLM3-wt sorafenib group compared with the HCCLM3-wt control, while sorafenib did not increase the number of lung metastases in the HCCLM3-IL-6(-) sorafenib group compared with the HCCLM3-IL-6(-) control. ($^{****}$P<0.0001 and P>0.05, respectively). The number of lung metastases in the HCCLM3-wt control was higher than that in the HCCLM3-IL-6(-) control ($^{****}$P<0.0001). IL-6, interleukin-6; IHMs, intrahepatic metastases; HCC, hepatocellular carcinoma; wt, wild-type.

analysis revealed that IL-6 induced EMT in HCCLM3 cells (Fig. 5B). As revealed in Fig. 5B, E-cadherin levels were higher in HCCLM3-IL-6(-) than in HCCLM3-wt cells, whereas the mesenchymal associated proteins vimentin and N-cadherin were downregulated in HCCLM3-IL-6(-) cells. These results indicated that the knockout of endogenous IL-6 could decrease the metastatic ability of HCCLM3 cells.

The IL-6 expression level was revealed to be low in isolated HepG2-wt supernatants (14). Therefore, to further examine the pro-metastatic and -EMT role of IL-6 in HepG2-wt cells, HepG2-wt cells were cultured in the presence of IL-6

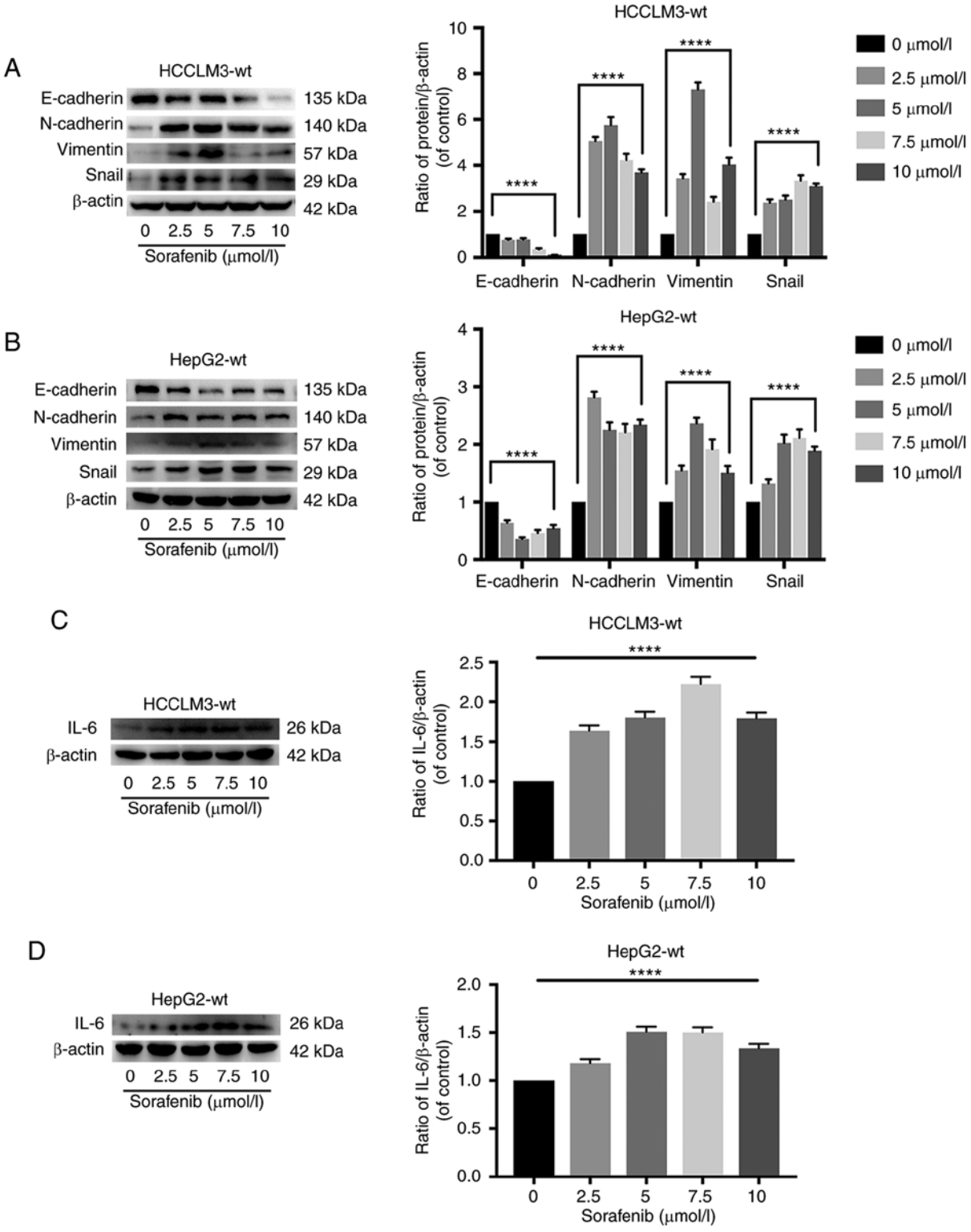


Figure 3. Sorafenib induces EMT and upregulates IL-6 in HCCLM3-wt and HepG2-wt cells, as revealed by western blot analysis. (A and B) E-cadherin was downregulated, and N-cadherin, vimentin and Snail were upregulated by sorafenib in HCCLM3-wt and HepG2-wt cells (all $^{****}P<0.0001$). (C and D) Sorafenib upregulated IL-6 in HCCLM3-wt and HepG2-wt cells (both $^{****}P<0.0001$). EMT, epithelial-mesenchymal transition; IL-6, interleukin-6; HCC, hepatocellular carcinoma; wt, wild-type.

to simulate the overexpression of the IL-6 gene in HepG2-wt cells. After 24 h, a Transwell assay was performed to evaluate the metastatic ability of HepG2-wt cells. In Fig. 5C, the metastatic ability of HepG2-wt cells cultured with exogenous IL-6 was greater than that of the control cells (Fig. 5C). Western blot analysis was performed to evaluate the markers associated with EMT: E-cadherin, N-cadherin, vimentin and Snail. Exogenous IL-6 induced EMT in HepG2-wt cells. As such, the levels of the epithelial marker E-cadherin were decreased, whereas those of vimentin and N-cadherin were significantly increased (Fig. 5D). The expression of Snail, a key regulator of EMT, was also increased following IL-6 treatment (Fig. 5D).

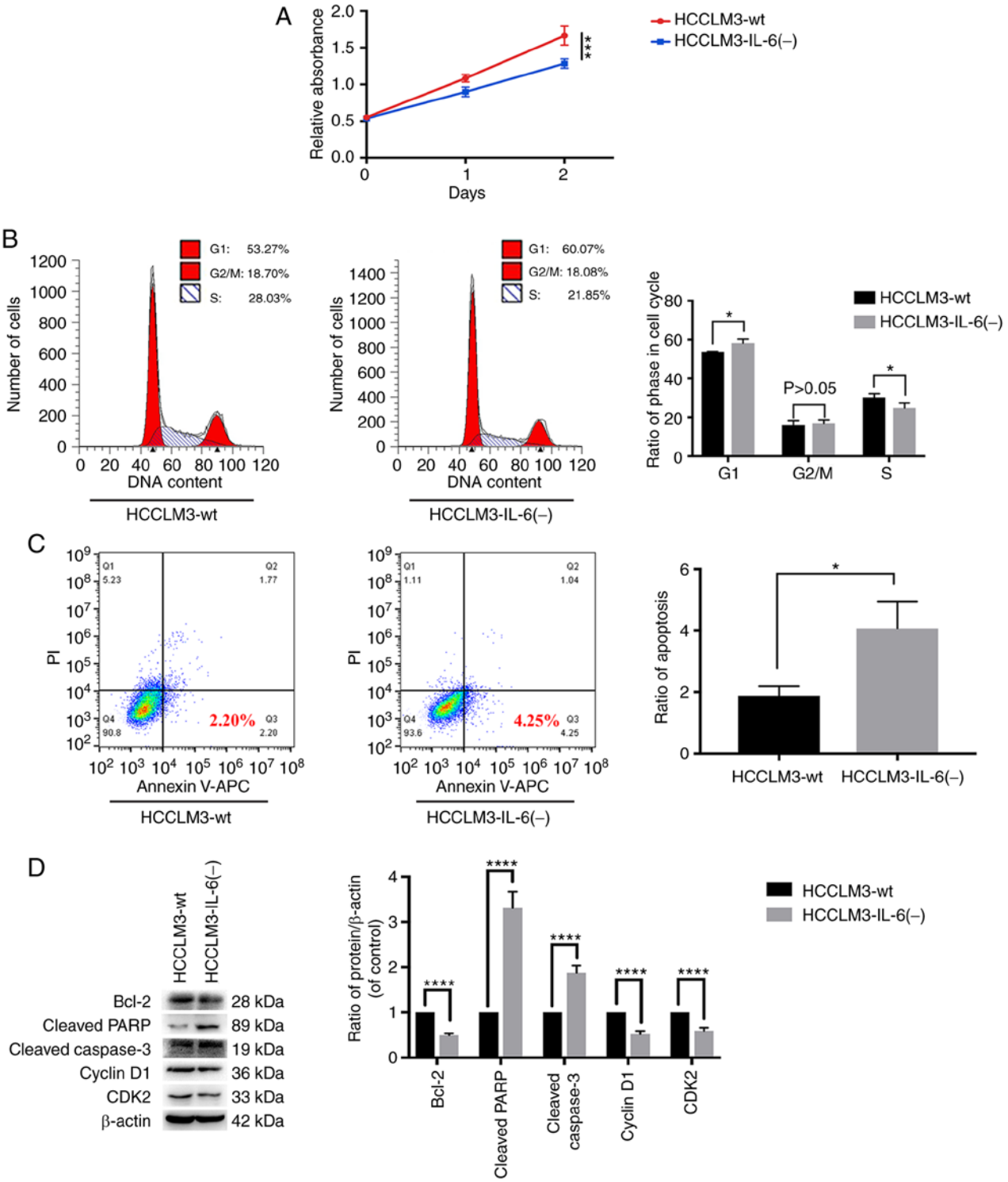


Figure 4. IL-6 knockout inhibits tumor cell growth, as revealed by CCK-8 assay, flow cytometric analysis and western blot analysis. (A) CCK-8 assay for cell proliferation of HCCLM3-wt and HCCLM3-IL-6(-) cells. IL-6 knockout inhibited liver cancer cell proliferation, as revealed by CCK-8 assay (***P<0.001). (B) Flow cytometric cycle assay of HCCLM3-wt and HCCLM3-IL-6(-) cells revealed that the knockout of IL-6 increased the proportion of cells at the G1 phase and decreased that of cells in the S phase (both *P<0.05). (C) Flow cytometric apoptosis assay of HCCLM3-wt and HCCLM3-IL-6(-) cells revealed that the knockout of IL-6 increased the cell apoptosis ratio as indicated by western blot analysis (*P<0.05). (D) Western blot analysis revealed that anti-apoptotic marker (Bcl-2) and cell cycle markers (cyclin D1 and CDK2) were downregulated in HCCLM3-IL-6(-) cells, as compared with HCCLM3-wt cells, whereas pro-apoptotic markers cleaved caspase-3 and cleaved PARP were upregulated in HCCLM3-IL-6(-) cells (all ****P<0.0001). CCK-8, Cell Counting Kit-8; wt, wild-type; IL-6, interleukin-6; HCC, hepatocellular carcinoma; Bcl-2, B-cell lymphoma-2.

HCCLM3-wt cells were also cultured with IL-6. The results of the Transwell assay and western blot analysis were consistent with HepG2-wt (Fig. S2). These results indicated that exogenous IL-6 promoted liver cancer metastasis and EMT.

*IL-6 knockout increases the susceptivity of HCCLM3 cells to sorafenib, as detected by CCK-8 assay, flow cytometric analysis and western blot analysis*. Furthermore, the proliferation inhibition and apoptosis induced by sorafenib in HCCLM3-wt

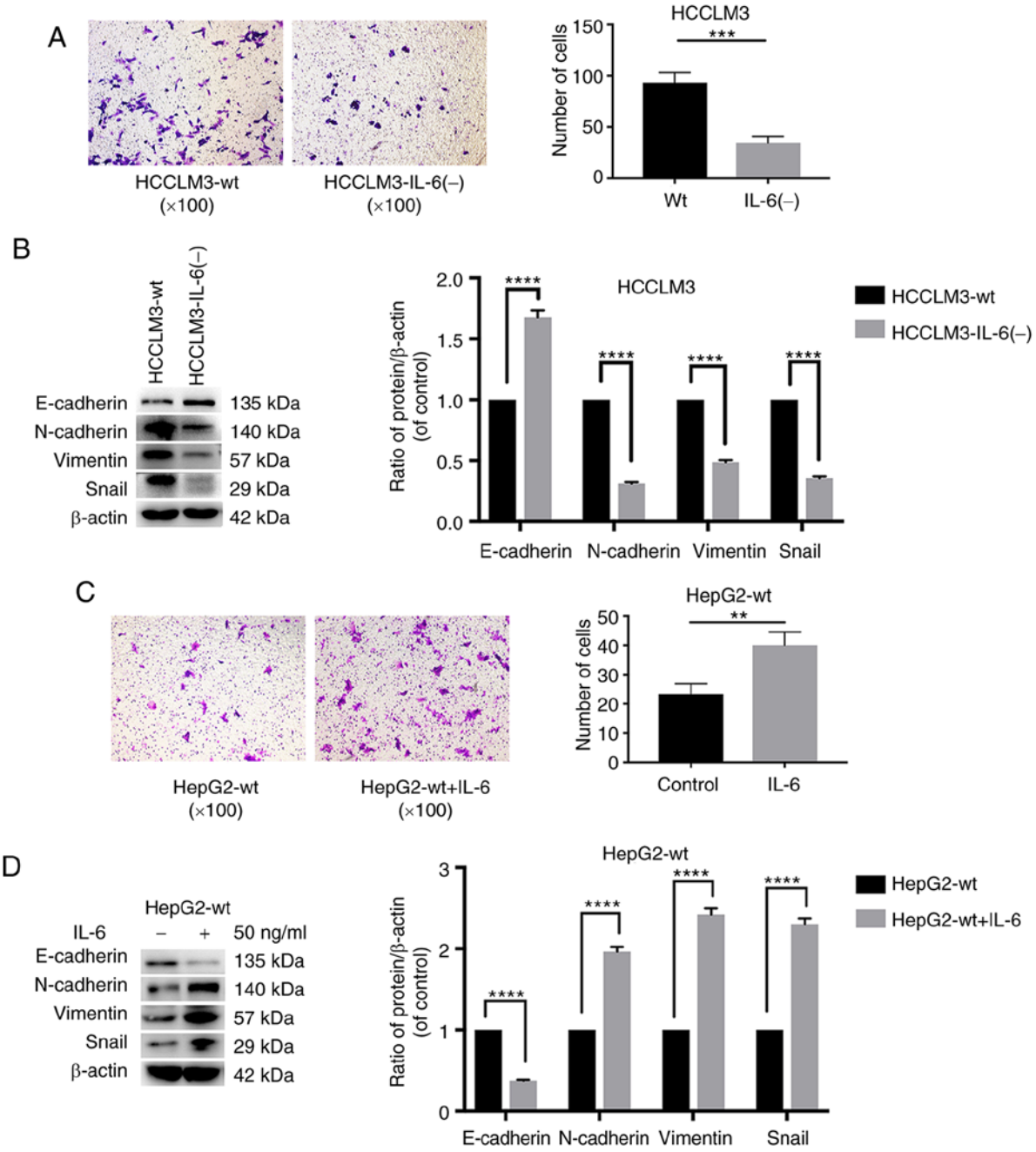


Figure 5. The knockout of IL-6 decreases the metastatic ability of HCCLM3 cells, and exogenous IL-6 increases the metastasis ability of HepG2 cells, as revealed by Transwell assay and western blot analysis. (A) The knockout of IL-6 decreased the metastatic potential of HCCLM3-wt cells, as revealed by Transwell assay (***P<0.001). (B) IL-6 knockout upregulated E-cadherin, and downregulated N-cadherin, vimentin and Snail in HCCLM3-IL-6(-) cells, as compared with HCCLM3-wt cells (all ****P<0.0001). (C) Exogenous IL-6 increased the metastatic ability of HepG2-wt cells as revealed by Transwell assay (**P<0.01). (D) Exogenous IL-6 downregulated E-cadherin, and upregulated N-cadherin, vimentin and Snail in HepG2-wt cells (all ****P<0.0001). IL-6, interleukin-6; HCC, hepatocellular carcinoma; wt, wild-type.

and HCCLM3-IL-6(-) cells was detected. When IL-6 was knocked out, the tumor cells were prone to an inhibited proliferation (Fig. 6A and B). In addition, flow cytometry was conducted to detect the apoptosis of HCCLM3 cells treated with sorafenib, and it was revealed that HCCLM3-IL-6(-) was prone to sorafenib-induced apoptosis (Fig. 6C and D). In addition, western blot analysis was performed to investigate the level of cell cycle (CDK2 and cyclin D1) and apoptotic (cleaved caspase-3, cleaved PARP and Bcl-2) markers. Following the administration of sorafenib, the level of cleaved caspase-3 and cleaved PARP in HCCLM3-IL-6(-) cells was higher than that in HCCLM3-wt cells (Fig. 6E). In addition, the level of Bcl-2 in HCCLM3-IL-6(-) cells was lower than that in HCCLM3-wt cells (Fig. 6E). In addition, the level of cell cycle markers, such as CDK2 and cyclin D1, in HCCLM3-IL-6(-) cells was lower than that in HCCLM3-wt cells (Fig. 6E). These results indicated that the knockout of endogenous IL-6 could increase their susceptibility to sorafenib.

*IL-6 induces EMT in liver cancer cells and promotes proliferation through JAK/STAT3/Snail pathway hyperactivation.* The hyperactivation of JAK/STAT3/Snail signaling has

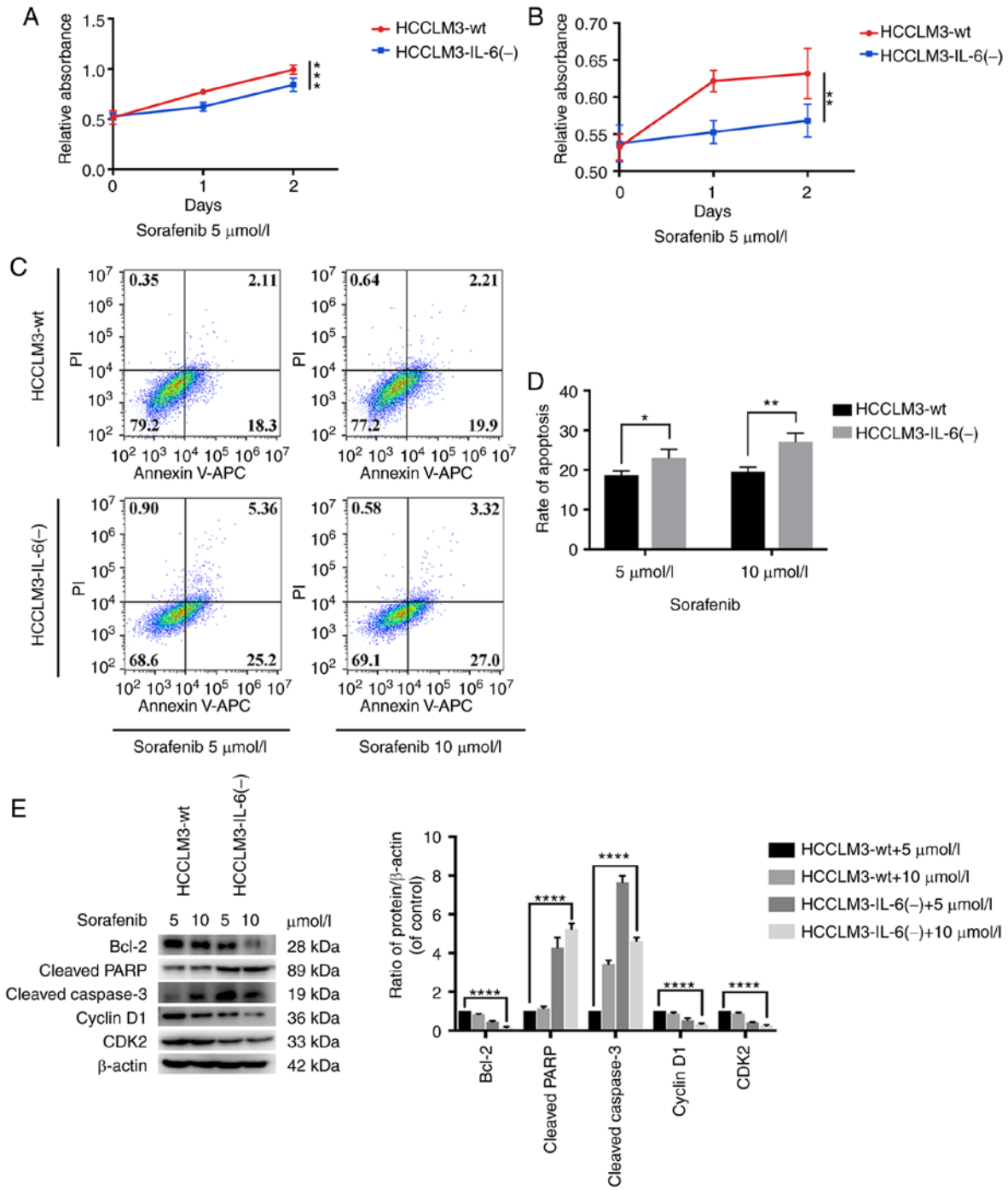


Figure 6. IL-6 knockout increases the susceptivity of HCCLM3 cells to sorafenib, as revealed by CCK-8 assay, flow cytometric analysis and western blot analysis. (A and B) CCK-8 assays for cell proliferation of HCCLM3-wt and HCCLM3-IL-6(-) cells revealed that the knockout of IL-6 increased the growth inhibition effect induced by 5 and 10 $\mu$mol/l sorafenib (***P<0.001 and *P<0.05, respectively). (C and D) Flow cytometric apoptosis assay of HCCLM3-wt and HCCLM3-IL-6(-) cells revealed that the knockout IL-6 increased the apoptosis induced by 5 and 10 $\mu$mol/l sorafenib (*P<0.05 and **P<0.01, respectively). (E) Western blot analysis revealed that the level of anti-apoptotic marker Bcl-2 and cell cycle markers cyclin D1 and CDK2 were lower in HCCLM3-IL-6(-) than HCCLM3-wt cells, whereas pro-apoptotic markers cleaved caspase-3 and cleaved PARP were higher in HCCLM3-IL-6(-) than in HCCLM3-wt cells following the administration of 5 and 10 $\mu$mol/l sorafenib (all ****P<0.0001). HCC, hepatocellular carcinoma; wt, wild-type; Bcl-2, B-cell lymphoma-2; IL-6, interleukin-6.

been revealed to be responsible for IL-6-induced EMT and proliferation (25). The aim of the present study was to test this hypothesis by inhibiting JAK/STAT3 signaling through AG490 (10 $\mu$mol/l), which is an inhibitor of JAK2 protein tyrosine kinase. Exogenous IL-6 was significantly increased in p-JAK2, p-STAT3 and Snail in HepG2 cells (Fig. 7A and B). The combination of IL-6 and AG490 exhibited the distinctly blocked JAK2 and STAT3 phosphorylation and inhibited the upregulation of Snail (Fig. 7A and B). IL-6 knockout significantly decreased p-JAK2, p-STAT3 and Snail in HCCLM3 cells (Fig. 7C and D). In addition, a CCK-8 assay revealed that exogenous IL-6 could promote HepG2-wt cell proliferation, while this effect was significantly blocked by AG490 (Fig. 7E). These results indicated that IL-6 may induce EMT and promote proliferation by activating JAK/STAT3/Snail signaling.

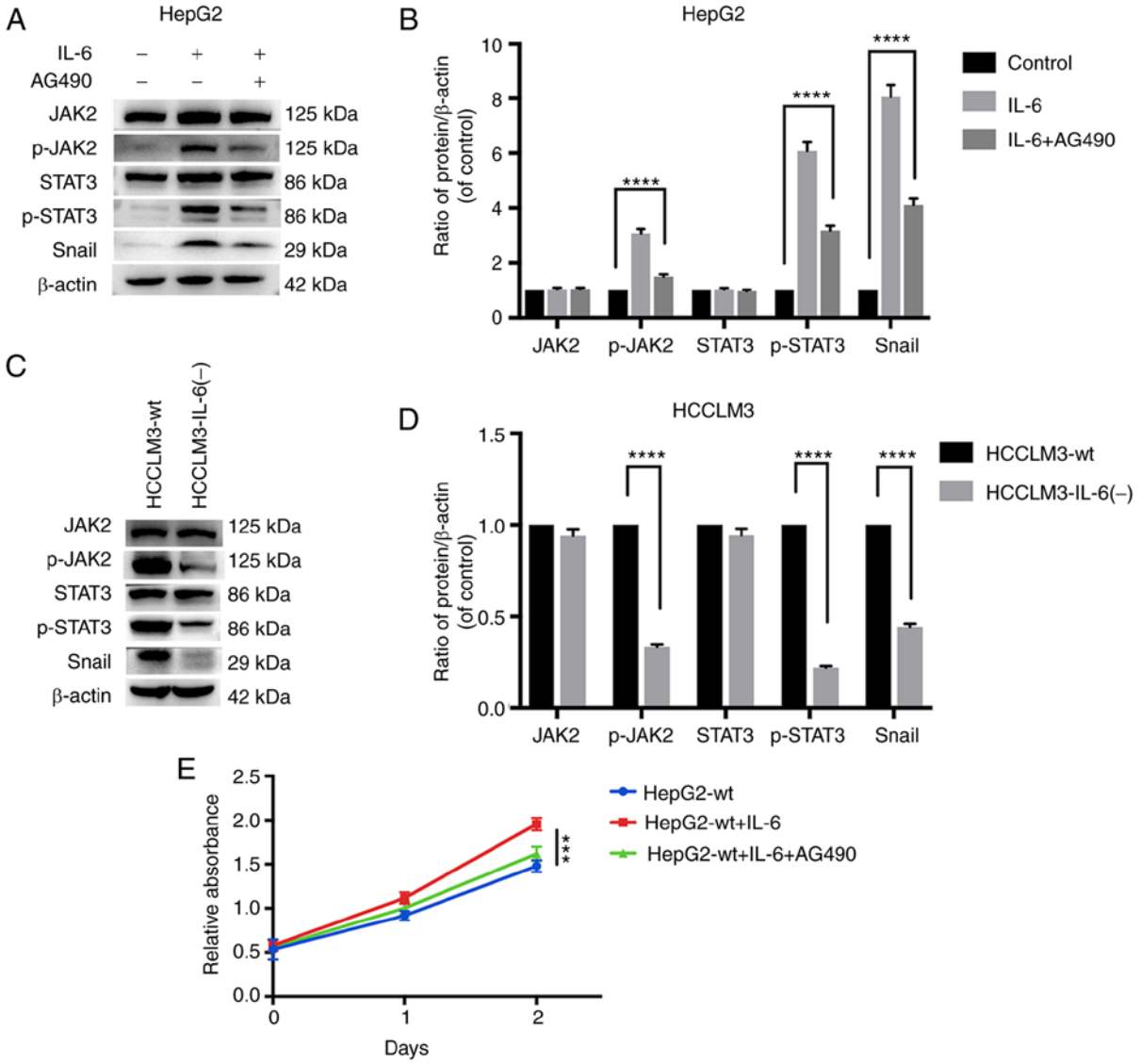


Figure 7. IL-6 induces liver cancer EMT through JAK/STAT3/Snail pathway hyperactivation. (A and B) Exogenous IL-6 hyperactivation of p-JAK2, p-STAT3 and increased Snail expression in HepG2-wt cells, whereas AG490 blocked the effect induced by IL-6 (all $^{****}$P<0.0001). (C and D) IL-6 knockout decreased p-JAK2, p-STAT3 and Snail expression in HCCLM3-IL-6(-) cells, as compared with HCCLM3-wt cells (all $^{****}$P<0.0001). (E) CCK-8 assay revealed that exogenous IL-6 could promote HepG2-wt cell proliferation, while this effect was significantly blocked by AG490 ($^{***}$P<0.001). IL-6, interleukin-6; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; wt, wild-type.

## Discussion

Sorafenib is multi-targeted tyrosine kinase inhibitor (TKI) that affects numerous signal pathways (5). Sorafenib leads to the blocking of key signaling pathways, namely, Ras/Raf/MAPK and PI3K/Akt/mTOR, which have been implicated in the pathogenesis of liver cancer (32,33). However, there remain numerous mechanisms that have not been revealed. As a first-line treatment drug, sorafenib has been revealed to inhibit tumor growth and prolong patient survival (6,7). However, the drug has been revealed to elicit several side effects and promote the invasive and metastatic potential of cells, as demonstrated by western blot analysis and *in vivo* assessment (9,34). Sorafenib has also been revealed to induce EMT in patients with liver cancer (35). Some patients with liver cancer quickly develop resistance to sorafenib with discontinued treatment (5). Some patients with renal cancer experience tumor recurrence and succumb after discontinuing sorafenib treatment (8). Therefore, understanding how sorafenib interacts with other treatments may be necessary to improve its efficacy and attenuate its side effects. The present study, to the best of our knowledge, is the first to reveal that sorafenib may affect tumors through the upregulation of the IL-6/STAT3 signaling pathway. The effect of IL-6 as a single factor on liver cancer was not only studied, but also the effect of IL-6 combined with sorafenib on liver cancer, to further demonstrate that sorafenib may induce tumor metastasis and chemotherapy resistance through the IL-6/STAT3 signaling pathway. Sorafenib could upregulate the expression of IL-6, and IL-6-induced EMT and tolerance to sorafenib may be a 'side effect' of sorafenib. Therefore, it is proposed that combined anti-IL-6/STAT3 may improve the efficacy of sorafenib.

EMT plays a critical role in tumor progression, especially in tumor invasion, metastasis and drug resistance. During tumor progression, epithelial cells gradually lose their features, such as the downregulation of E-cadherin, and obtain mesenchymal

characteristics, such as the upregulation of N-cadherin and vimentin (23,36,37). Snail has been identified as a key regulator of EMT during embryonic development and cancer progression, and it is effective in inhibiting E-cadherin expression and enhancing tumor invasion and metastasis (18,38). The present results revealed that sorafenib promoted liver cancer cell metastasis and induced EMT. Sorafenib treatment increased IL-6 expression in liver cancer cells.

IL-6 is a pleiotropic cytokine present in the tumor microenvironment; it is associated with poor prognosis, recurrence and metastasis in various types of cancer (10,39). Plasma levels of IL-6 and its soluble receptor were associated with cancer progression and bone metastasis in prostate cancer (40). Sullivan *et al* (24) revealed that exogenous IL-6 exposure increased breast cancer cell metastasis and induced EMT in MCF-7 cells that do not express IL-6. To verify the function of IL-6 in liver cancer, we not only exposed liver cancer cells to exogenous IL-6, but also knocked out the endogenous IL-6 of liver cancer cells. The present results revealed that either endogenous or exogenous IL-6 affected the metastatic ability and EMT progression of liver cancer cells. The disrupted IL-6 expression in liver cancer cells markedly attenuated the pro-invasive effect of sorafenib treatment, as detected by western blot analysis and *in vivo* assessment. Intrahepatic invasion and lung metastasis are poor prognostic indicators for patients with liver cancer (41,42). In the present study, it was revealed that IL-6 knockout in HCCLM3 cells could decrease the number of lung metastasis but not intrahepatic invasion *in vivo*, probably due to the trait of the HCCLM3 cells, which originate from nude mouse lung metastasis with a highly distant metastatic potential (43).

The chemical resistance of cancer cells to conventional chemotherapy and targeted drugs is the main disadvantage of current chemotherapeutic strategies for various types of tumors, including liver cancer (44). Zhang *et al* (45) revealed that EMT is responsible for sorafenib resistance. The present results also indicated that sorafenib resistance may be associated with IL-6-mediated EMT. IL-6 plays a vital role in trastuzumab resistance in HER2/neu positive breast cancer, which mediates the expansion of cancer stem cells by downregulating PTEN expression and triggering Akt and STAT3, thereby leading to nuclear factor-κB activation (46). It was revealed herein by western blotting that IL-6 knockout increased the apoptosis induced by sorafenib in liver cancer cells. These findings indicated that an IL-6 signaling network may be a potential therapeutic target for liver cancer and a biomarker for predicting the response to sorafenib treatment.

Some contradicting findings have yet to be elucidated. It was revealed by western blot analysis that IL-6 affected tumor cell proliferation and apoptosis. The disruption of IL-6 expression in liver cancer cells increased the susceptibility to sorafenib *in vitro*. IL-6 knockout did not influence the volume of tumors in a xenograft model of nude mice. In addition, IL-6 knockout decreased the number of lung metastatic nodules but did not affect the number of IHM. Possible reasons include the following: First, the tumor microenvironment is a complex environment, IL-6 is secreted by various cells in the tumor environment, including cancer cells, tumor-associated fibroblasts, macrophages and cancer stem cells (10). Although the IL-6 gene is knocked out in tumor cells, other cells in the microenvironment can still secrete and affect tumor cell growth. However, in distant metastasis locations, such as the lung, no inflammatory environment is formed at the beginning. The source of IL-6 is mainly from the tumor itself. Therefore, knocking out the IL-6 gene in tumor cells may have a greater affect in lung metastasis than intrahepatic metastasis. Moreover, tumor-associated macrophages (TAMs) play a crucial role in liver cancer progression, growth and invasiveness (47,48). IL-6 was revealed to be undetectable in isolated HepG2-wt supernatants and had a low expression in TAM supernatants, whereas co-cultured HepG2-wt cells and TAMs increased IL-6 expression 10 times more than cultured HepG2-wt alone (14). Aside from tumor cells, tumor microenvironments should also be considered. Therefore, CNTO 328, a humanized monoclonal antibody targeting IL-6, which can systemically neutralize IL-6 bioactivity, should be further investigated with sorafenib in liver cancer.

The present study has certain limitations. First, the molecular mechanism between IL-6 and sorafenib resistance should be further explored. Studies should verify whether IL-6 overexpression or knockdown in various liver cancer cells is associated with sorafenib resistance. Secondly, IL-6 is a poor prognostic factor of patients with liver cancer (14-16). However, the association between IL-6 and sorafenib treatment in patients remains unclear. Thirdly, aside from the JAK/STAT pathway, the ERK1/2/MAPK and PI3K/Akt pathways can be activated by IL-6, and may potentially account for IL-6-mediated EMT and resistance (49).

In conclusion, the present findings demonstrated that sorafenib promoted the tumor metastasis potential via IL-6-mediated EMT by activating JAK2/STAT3 signaling. CNTO 328 a promising antibody-drug conjugate targeting cytokine IL-6, has been tested in clinical trials of several cancer models, including renal cell cancer, ovarian cancer and multiple myeloma and a direction of our future research will be to assess it in liver cancer (50-52). The present results provided novel insights into the role of IL-6 in liver cancer and emphasized that the efficiency of a sorafenib-based strategy may be improved by combining it with anti-IL-6 therapies for liver cancer.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

JS and PYZ designed the study and conducted a critical revision of the manuscript. KWZ and DW conducted the cell viability, flow cytometry, western blotting, RT-PCR and migration assays. KWZ, HC and MQC conducted the animal

experiments. DW and YYZ were responsible for the collection and assembly of data. KWZ and DW prepared the figures and wrote the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

All animal experiments were approved by the Animal Care Committee of Zhongshan Hospital (Shanghai, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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