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# Cepharanthine suppresses proliferation and metastasis and enhances apoptosis by regulating JAK2/Stat3 pathway in hepatocellular carcinoma

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**ABSTRACT**

Hepatocellular carcinoma (HCC) is a familiar malignant tumor, and cepharanthine (CEP) was proven to prevent the malignant activity of multiple cancer cells, including HCC. However, there are few reports on the regulatory role of CEP in HCC. After treatment with CEP or/and JAK2/Stat3 inhibitor (AG490), the associative functions were assessed by MTT, wound healing, Trans well, and Hochest33342-PI double staining in HCC cells. Then the levels of CDK4, MMP-9, Bcl-2, p-JAK2/JAK2, and p-Stat3/Stat3 were monitored via western blot. Besides, the HCC xenograft model was constructed to verify the effects of CEP on tumor growth and the JAK/Stat3 pathway. CEP could restrain proliferation and metastasis and facilitate apoptosis in HCC cells. CEP also reduced Bcl-2 (anti-apoptosis), CDK4 (proliferation), and MMP-9 (invasion) expressions, and inhibited JAK2 and Stat3 phosphorylation. Besides, CEP suppressed HCC progression by JAK2/Stat3 pathway. Moreover, CEP inhibited the growth of subcutaneous HCC xenografts and reduced p-JAK2 and p-Stat3 in tumor tissues. CEP could suppress HCC progression by attenuating the JAK2/Stat3 pathway, indicating that CEP might be a therapeutic drug for HCC patients.

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## Introduction

Primary liver cancer is the seventh leading cancer (1). And hepatocellular carcinoma (HCC) accounts for about 75% of liver cancers, and hepatitis B virus infection is the most frequent risk factor for its development; it also includes hepatitis C virus infection, nonalcoholic hepatitis, fatty liver, and other genetic disorders (2,3). The prognosis of HCC patients depends on the tumor stage, with a 5-year survival rate of 50% to 70% after radical treatment in early-stage patients. Patients with progressive disease lose the opportunity for radical therapy and have a 3-year survival rate of only 10% to 40% (4). However, there are no specific symptoms in the early stages of HCC, and most patients with HCC are already in the middle and late stages of the disease, resulting in a poor prognosis for patients (5). Therefore, early diagnosis and therapy is the key to improving the prognosis of patients.

Based on the actual situation of patients, Traditional Chinese medicine (TCM) can treat patients based on syndrome differentiation, addition, and reduction of drugs (6). TCM also can achieve the effects of regulating the Yin and Yang of the human body and anti-tumor through the treatment of tonifying Qi, soothing the liver and promoting blood circulation (7). In TCM, HCC is based on qi and blood deficiency, liver loss and dispersion as the basic pathogenesis, and the interconnection of qi, blood, dampness, heat, stasis and poison as the standard (8). In different stages of HCC, strengthening the body's resistance to eliminate pathogenic factors is performed through dialectic treatment methods, such as soothing the liver and regulating qi, activating blood circulation and removing blood stasis, clearing heat and detoxification, and the dialectical method of treating both symptoms and root causes are applied to restore the function of liver controlling dispersion, and allow qi and blood flow, and eliminating dampness, heat and stasis toxins (9). This has obvious advantages in controlling symptoms, improving immunity, reducing metastasis, and improving the quality of survival. Cepharanthine (CEP) is the active ingredient extracted from *Stephania*, frequently used to treat various acute and chronic diseases (10). CEP has also been used for alopecia areata, xerostomia, snakebite, circumsolar alopecia, sarcoidosis, refractory anemia, etc (11). Besides, CEP has anti-inflammatory, antibacterial, anti-parasite, and immunity-boosting properties (12-14). Currently, CEP has been reported to prevent cancer progression in multiple cancers, such as breast cancer (15), ovarian cancer (16), and colorectal cancer (17), etc. Research also verified that CEP could suppress proliferation and enhance apoptosis of HCC cells (18). However, the mechanism by which CEP blocks the progression of HCC is not fully understood.

The JAK2/Stat3 pathway is aberrantly expressed in many types of tumor cells (19,20). The JAK2/Stat3 pathway can also participate in various physiological activities, such as cell proliferation, apoptosis, and immune function in different organs, including the liver (21). Stat3 is a key component of the JAK2/Stat3 pathway, and phosphor-activated Stat3 can affect aberrant proliferation and metastasis of cancer cells (22). Therefore, blocking the JAK2/Stat3 pathway can be a direction to treat malignant tumors. However, whether CEP can alter the HCC process

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by JAK2/Stat3 pathway has not been reported.

In this study, we further investigated whether CEP can affect the proliferation, apoptosis, and metastasis of HCC cells by regulating the JAK2/Stat3 pathway, to provide a reference for HCC therapy with CEP.

## Materials and Methods

### Cell culture

Mouse normal liver cells (AML-12), and HCC cells (HepG2 and SMMC-7721) were bought from Shanghai Cell Bank, Chinese Academy of Sciences. All three cells were cultured in DMEM/F12 (Gibco, Grand Island, NY, USA) with 10% FBS (Gibco, Rockville, MD, USA) at 37℃ with 5% CO2.

### Cell treatment

First, AML-12 and HCC cells were processed with 0, 5, 10, and 20 μM CEP (Manst Biotechnology, Chengdu, China) for 24 h, respectively. Second, HCC cells were also disposed of 0, 40, 80, 120, and 160 μM JAK2/Stat3 inhibitor (AG490, MedChem Express, NJ, USA) for 24 h. Third, HCC cells were addressed with 10 μM CEP or/and 160 μM AG490 for 24 h.

### MTT

AML-12 and HCC cells (6×103 cells/well) were inoculated uniformly in a 96-well plate, and sterile PBS (Gibco) was added to the edge wells. Then the cells were added with CEP or AG490, incubated for 24 h and added with 100 μL MTT (1mg/mL, Promega, Madison, WI, USA). After 4 h, cells were immersed in 150 μL DMSO. Then the absorbance was examined with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm and cell viability was calculated.

### Hochest33342-PI double staining

HCC cells were inoculated in 96-well plates (1000 cells/well) and incubated with 5, 10, 20 μM CEP for 8 h. After washing, the cells were addressed with 50 μL Hochest33342 solution at 37°C with 5% CO2 for 30 min. After washing, cells were disposed of 1μg/ml PI solution at 37°C for 1 min. After washing, the results were observed by fluorescence microscopy (Nikon Instruments Inc., NY, USA), and the apoptosis rate was calculated after counting.

### Wound healing

The well-grown cells were placed in 6-well plates with an inoculum of about 5×105 cells. On day 2 or 3 (cell density of approximately 90 %), cells were drawn in a straight line with a 200 μl gun tip. After washing, cells were cultured with the serum-free medium. Then cells were photographed with a microscope at 0, 24, and 48 h. The migration rate of cells in different groups at different times was also calculated.

### Transwell

Matrigel was mixed with DMEM/F12 complete medium at a dilution of 1:4. And 50 μ1 of Matrigel was spread in Transwell chambers (8 μm, Costar, Inc., CA, USA) for 30 min at 37°C. HepG2 and SMMC-7721 cells (500 μL/well) were added to the upper chamber of Transwell chambers at 3×105/well and 1×105/well with free FBS and different concentrations of CEP, respectively. 600 μL of medium with 10% FBS was plated into the lower chamber for 24 h. After removing the upper layer of cells, the invaded cells were fixed and stained, washed with PBS, and counted with an inverted microscope.

### Western blot

Groups of cells were harvested and were lysed with RIPA (Beyotime, Ningbo, China) for 30 min. After centrifugation, the supernatant was collected. After quantification, the protein was denatured, separated with 10% SDS-PAGE by electrophoresis, and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After closure with 5% skim milk powder for 2 h, the membranes were exposed to diluted primary antibodies at 4°C overnight and diluted secondary antibodies (Abcam, Cambridge, MA, USA) for 1.5 h. The protein bands on the membranes were tested by ECL kit, and the protein level was analyzed by graphing. The primary antibodies (p-Stat3 (Tyr 705) and Stat3) were from Abcam (Cambridge, MA, USA); JAK2, p-JAK2 (Tyr 1007), CDK4, Bcl-2, and GAPDH were from Cell Signaling Technology (Beverly, MA, USA); and MMP-9 was from Affinity (JiangSu, China).

### Animal

SPF-grade male BALB/C-NULL nude mice (4-6 weeks, weighing 18-22 g, No. SCXK (Guangdong) 2020-0167) were purchased from Southern Medical University (Guangzhou, China). The nude mice were housed in the SPF-grade Laboratory (No. N0.44002 I000I2689). The experimental animals were housed and operated following the ethical requirements of the Animal Ethics Committee of Southern Medical University.

### Subcutaneous tumor experiment in nude mice

SMMC-7721 cells were harvested, made into single-cell suspensions, and mixed with Matrigel in a 1:1 ratio. SMMC-7721 cells (5×106 cells) were injected subcutaneously into the right shoulder of nude mice. After one week, tumorigenesis was observed. Once nodules were found under the skin of the nude mice, the step of gavage was performed. Mice were divided into control (mice were given 0.2 ml normal saline with 5% Tween-80 by gavage), CEP-10mg/kg (mice were given 0.2 ml 10mg/kg CEP) by gavage, and CEP-10mg/kg groups (mice were given 0.2 ml 20mg/kg CEP by gavage). The tumor size and body weight of nude mice were examined every 4 days. Approximately 0.6-1 ml of blood was taken from the heart. After resting for 30 min-1 h, the whole blood was placed into a centrifuge for centrifugation (3000 rpm for 15 min). and the serum was collected and stored. Then the subcutaneous tumors from nude mice were weighed and immersed in 4% paraformaldehyde for 24 h. The tissues were trimmed and made into paraffin sections.

### Immunohistochemistry (IHC)

Paraffin sections of each group were dehydrated by ethanol gradient method and antigen retrieval was performed with 0.01M sodium citrate. After blocking with goat serum, each section was added with the primary antibody (p-JAK2, Cleaved-Caspase-3, CDK4; diluted 1:100) at 4°C overnight and HRP-labeled secondary antibodies at 37°C for 30 min. Then the sections were stained using DAB, counterstained with hematoxylin, differentiated,

reverse blue, hydrated, transparent, and sealed, and observed.

### Statistical analysis

The data were displayed as x ± s, and the data was analyzed using SPSS21.0 software. One-way ANOVA was adopted for comparison, and the LSD method was applied if the variance was equal. If the variances were uneven, Welch robust estimation was selected, followed by Dunnett's T3 for pairwise comparisons. P<0.05 means the result is meaningful. Graphpad Prism8 was applied to make bar graphs.

## Results

### CEP suppresses the malignant progression of HCC cells

To probe the impacts of CEP on the related functions of HCC cells, different concentrations of CEP were applied to induce HCC and AML-12 cells. MTT data indicated that cell viability was markedly decreased in CEP-treated HCC cells, and with the gradual increase of CEP concentration, the cell viability rate appeared to a decreasing trend; while CEPhad little effect on the cell viability of normal liver cells (AML-12) (Figure 1A). Meanwhile, results denoted that the PI-positive cells were observably increased in CEP-treated HCC cells, and the apoptosis ability was gradually enhanced with the increase of CEP concentration (Figure 1B). Besides, wound healing results suggested that CEP treatment memorably suppressed the scratch healing ability of HCC cells, and the scratch healing ability demonstrated a gradual weakening trend with the gradual acceleration of CEP concentration (Figure 1C). Consistently, the Transwell results presented that the invasive cells were dramatically reduced in CEP groups, and the invasive ability notably diminished with the rising of CEP concentration (Figure 1D).

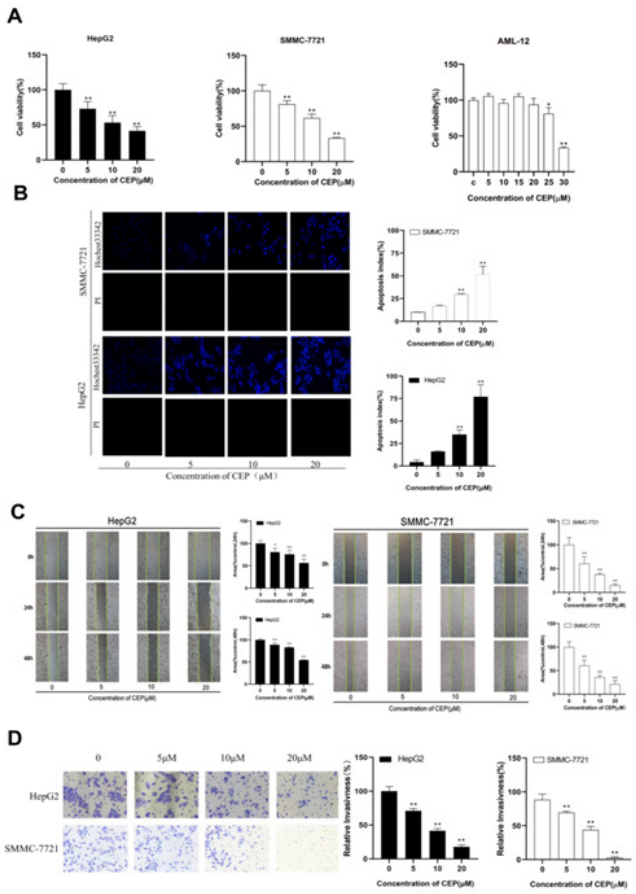


**Figure 1.** CEP restrains proliferation and metastasis and heightens apoptosis in HCC cells. Human normal liver cells (AML-12), and HCC cells (HepG2 and SMMC-7721) were processed with 0, 5, 10, and 20 μM CEP. (A) Cell viability was examined through MTT assay in CEP-treated AML-12, HepG2, and SMMC-7721. (B) Hochest33342-PI double staining was adopted to test the change in the apoptosis of the processed HCC cells. (C) Wound healing was utilized to analyze cell migration ability in the HCC cells. (D) Transwell denoted the changes in an invasion of HCC cells. **P<0.01.

### CEP down-regulates CDK4, MMP-9, and Bcl-2 in HCC cells

Meanwhile, we also monitored the levels of proliferation (CDK4), metastasis (MMP-9), and anti-apoptosis (Bcl-2) related proteins, and found that CDK4, MMP-9, and Bcl-2 expressions were prominently reduced in CEP treatment groups (especially 20 μM CEP) (Figure 2A-2F).

### CEP weakens the JAK/Stat3 pathway in HCC cells

To further elucidate the mechanism of the anti-tumor activity of CEP in HCC cells, we evaluated the phosphorylation of JAK2 and Stat3 after induction with CEP. The data disclosed that the p-JAK2 level was distinctly lessened in CEP groups (especially 20 μM CEP) versus that in the control group (Figures 3A and 3B). Similarly, CEP also downregulated p-Stat3 in HCC cells, especially 20 μM CEP (Figure 2G and 2J).

### CEP prevents malignant behavior by the JAK2/Stat3 pathway in HCC cells

To further investigate whether CEP could restrain HCC progression by JAK2/Stat3 pathway, we first applied different concentrations of JAK2/Stat3 inhibitor (AG490) to induce HCC cells, and the results exhibited that relative to the control group, cell viability was outstandingly reduced in AG490 groups, and cell viability of HCC cells reached the lowest when the concentration of AG490 was 160 μM (Figure 3A). Then CEP-treated HCC cells were also disposed of with JAK2/Stat3 inhibitor (AG490). The results displayed that both CEP and AG490 memorably could diminish the viability of HCC cells, and AG490 also could further encourage the repressive role of CEP on the viability of HCC cells (Figure 3B). Additionally, Hochest33342-PI double staining results denoted that both CEP and AG490 could accelerate apoptosis, and both had a synergistic effect on the induction of apoptosis in HCC cells (Figure 3C). Consistently, the Transwell data denoted the invasion capacity of HCC cells could be notably suppressed by CEP or AG490, and the invasiveness of HCC cells could be further diminished when CEP and AG490 were combined to treat (Figure 3D).

### CEP downregulates CDK4, MMP-9, and Bcl-2 by the JAK2/Stat3 pathway in HCC cells

Consistently, the effects of CEP and AG490 on proliferation, metastasis, and apoptosis-related proteins were further validated in HCC cells. As displayed in western blotting data, relative to the control group, CDK4, MMP-9, and Bcl-2 levels were outstandingly down-regulated in the CEP or AG490 treatment group, and the down-regu-

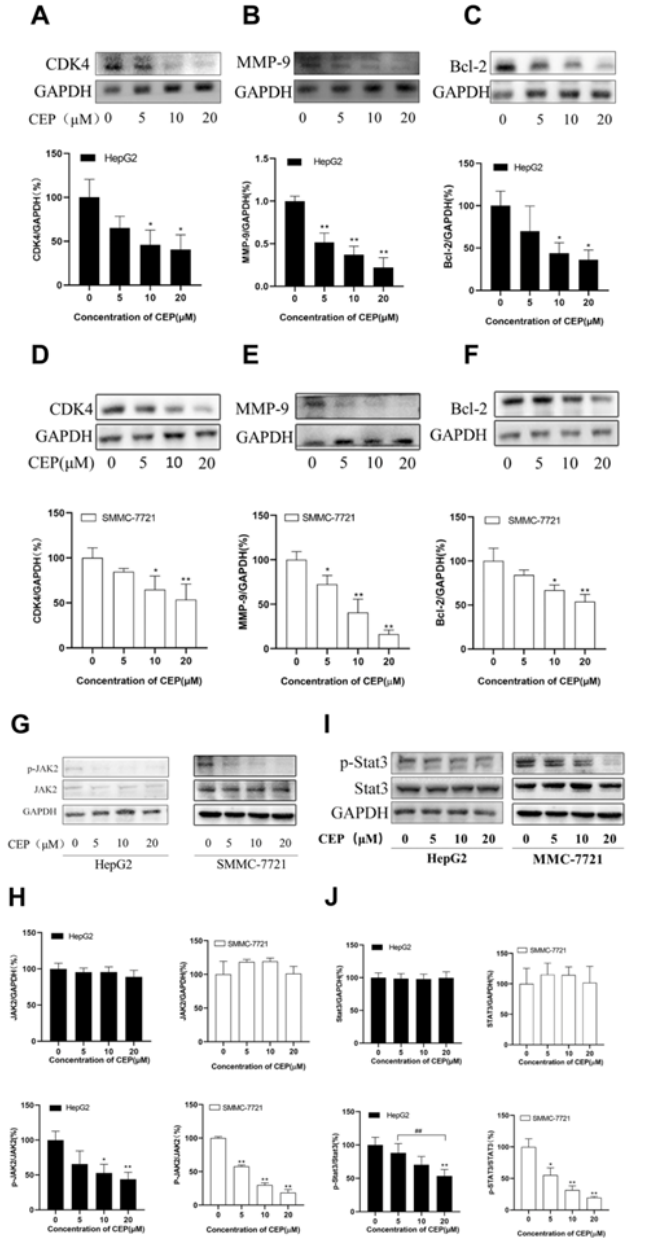


**Figure 2.** CEP down-regulates the expression of CDK4, MMP-9, and Bcl-2, as well as inhibits JAK/Stat3 pathway in HCC cells. After administration with CEP (0, 5, 10, and 20 μM), western blotting analysis of CDK4 (A), MMP-9 (B), and Bcl-2 (C) expressions in the treated HepG2 cells. And western blotting analysis for the evaluation of CDK4 (D), MMP-9 (E), and Bcl-2 (F) expressions in the treated SMMC-7721 cells. (G) The expression changes of p-JAK2 and JAK2 were credited by applying a western blot. (H) The ratio of p-JAK2 and JAK2 was quantified in line with the gray value. (I) Western blot revealed the expression changes of p-Stat3 and Stat3. (J) The ratio of p-Stat3 and Stat3 was also quantitatively analyzed. *P<0.05, **P<0.01.

lation effects of CEP and AG490 on these three proteins also showed an obvious synergistic effect (Figure 4A-4F). Thus, we further verified the inhibition of CEP on HCC progression, which may be achieved via the JAK2/Stat3 pathway.

## AG490 heightens the inhibiting effect of CEP on the JAK/Stat3 pathway in HCC cells

We further confirmed the impact of co-treatment of CEP and AG490 on the JAK2/Stat3 pathway. The data signified that CEP and AG490 could prominently down-regulate p-JAK2 and p-Stat3, respectively; and co-culture with AG490 also could distinctly heighten the inhibiting effect of CEP on the p-JAK2 and p-Stat3 expressions in HCC cells (Figure 4G-4J).

## CEP prevents tumor growth and promotes apoptosis *in vivo*

Finally, we further determined the role of CEP on the growth of HCC xenografts, and whether CEP could regulate the JAK/Stat3 pathway. HCC xenografts were treated with CEP by gavage. Through measurement and analysis, we obtained the growth curve of subcutaneous graft tumors. First, the data demonstrated that the tumor volume was conspicuously decreased in CEP groups (Figure 5A). Consistently, the tumor in each group was presented, and the weight of the tumor was dramatically reduced in CEP groups (Figure 5B and 5C). Additionally, during the whole experiment, the body weight of mice did not change (Figure 5D). Further, IHC results revealed that CEP treatment notably restrained p-JAK2 and CDK4 expressions, and memorably expedited cleaved caspase-3 expression in the tumor tissues of nude mice, and with the increase of CEP concentration, p-JAK2 and CDK4 expressions presented a downward trend, and the expression of cleaved caspase-3

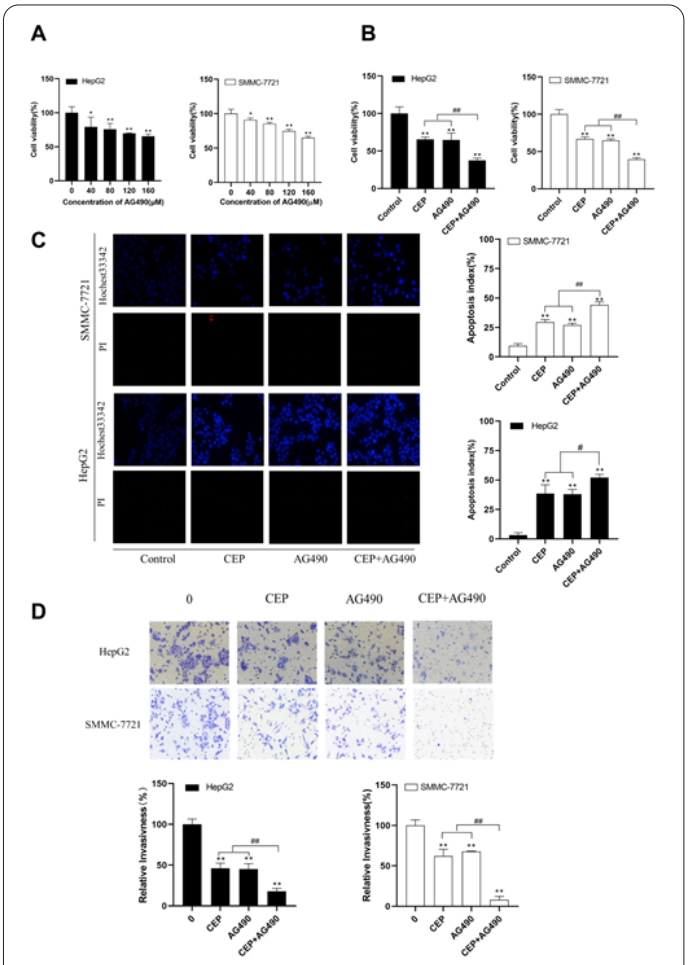


**Figure 3.** AG490 co-culture further enhances the effects of CEP on the attenuation of proliferation and invasion and the enhancement of apoptosis in HCC cells. (A) MTT analysis of the change of cell viability in HCC cells with 0, 40, 80, 120, and 160 μM AG490. (B) The cell viability was monitored through the application of MTT in HCC cells with 10 μM CEP or/and 160 μM AG490. (C) Hochest33342-PI double staining for the determination of apoptosis in HCC cells, the treatment was the same as in B. (D) Cell invasion was assessed by Transwell in CEP and AG490 treated HCC cells. *P<0.05, **P<0.01.

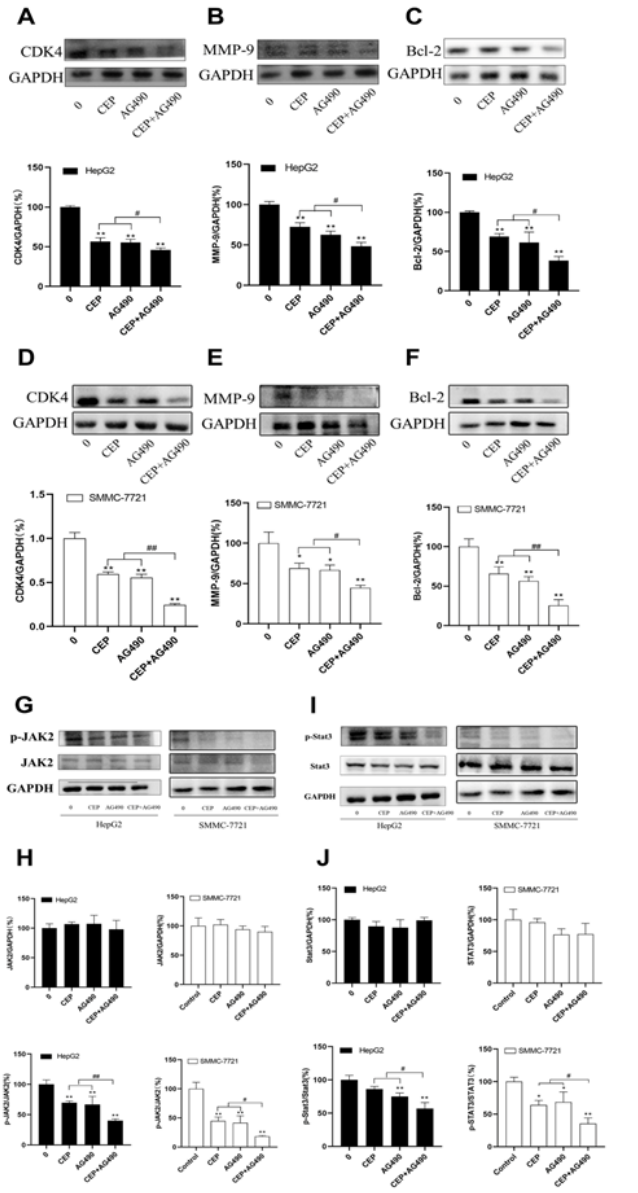


**Figure 4.** AG490 reinforces the lowering effect of CEP on CDK4, MMP-9, and Bcl-2 expressions, as well as the JAK/Stat3 pathway in HCC cells. HepG2 and SMMC-7721 cells were addressed with 10 μM CEP or/and 160 μM AG490. The expression changes of CDK4 (A), MMP-9 (B), and Bcl-2 (C) were identified using a western blot in the HepG2 cells. CDK4 (D), MMP-9 (E), and Bcl-2 (F) expressions were also confirmed by western blot in the SMMC-7721 cells. (G) After processing with CEP or/and AG490, a western blot was conducted to examine the change of p-JAK2 and JAK2 expressions in HCC cells. (H) Quantitative analysis of p-JAK2/JAK2. (I) Western blot for the confirmation of the expression changes of p-Stat3 and Stat3 in the treated HCC cells. (J) Quantitative analysis of p-Stat3/Stat3. *P<0.05, **P<0.01.

displayed an upward trend (Figure 5E).

## Discussion

HCC is highly malignant and prone to invasion and metastasis, and recurrence and metastasis are the major obstacles to HCC therapy (2,4). The clinical symptoms of liver cancer, such as "mass in the abdomen", "abdominal mass", "distention of abdomen", "hypochondriac" and "jaundice", are similar to the "accumulation" disease in the classic work of Chinese medicine "Nei Jing" (9). Thus, the treatment of HCC can play the unique efficacy and synergistic intervention of TCM, which is applied in clinical practice to prevent tumor progression and metastasis. CEP is an amphiphilic, positively charged alkaloid that increases the stability of the plasma membrane, improves immunity, and suppresses inflammation (23). CEP also has anti-tumor growth and metastasis effects (12,18). CEP also can induce apoptosis in cancer cells and reverse tumor drug resistance, thereby increasing the activity of chemotherapeutic agents (24). Thus, CEP has a stopping effect on the cancer process.

Homeostatic imbalance of cell proliferation and apoptosis is one of the pathogenesis of tumors (25). Therefore, it can slow down the development of HCC cells by inhibiting cell proliferation and accelerating apoptosis. A study has testified that CEP can prevent HCC cell proliferation and expedite cell cycle arrest and apoptosis (18). Most genetic events related to cell proliferation in the cell cycle occur in the G1 phase. Thus, the G1/S phase transition plays a key role in cell cycle progression. Cell cycle regulatory proteins such as CyclinD1 and related protein kinases (CDK2 and CDK4) are essential for cell cycle progression from the G1 phase to the S phase (26,27). Upregu-

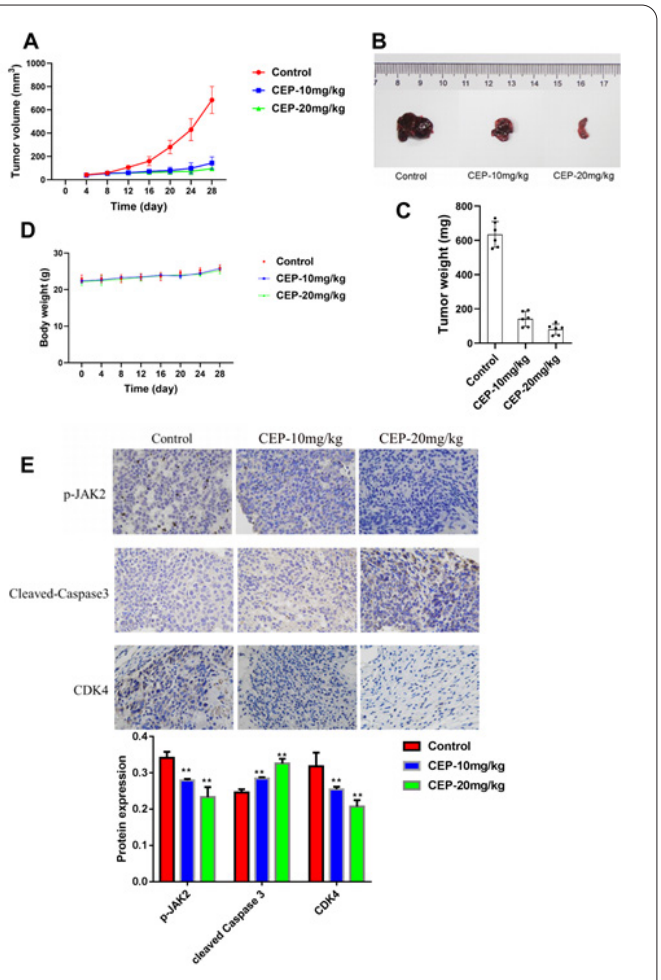


**Figure 5.** CEP prevents tumor growth and boosts apoptosis in vivo. The HCC xenografts model was first established usingSMMC-7721 cells, which were also treated with 10mg/kg and 20mg/kg CEP by gavage. (A) The volume of the transplanted tumor in nude mice was counted at 4-day intervals after intragastric administration. (B) The tumor in mice was collected and displayed at 28 days. (C) The weight of the transplanted tumor was measured at 28 days. (D) The nude mice were weighed every 4 days after intragastric administration. (E) IHC analysis of p-JAK2, cleaved-caspase-3, and CDK4.

lation of CDK4 expression can cause abnormal proliferation of HCC cells (28). The study also stated that CEP has the function of inducing cell arrest in ovarian cancer cells during the Gl and S phases of the cell cycle (16). Bcl-2 and Bax are apoptosis-regulating proteins that form a dimer but antagonize each other (29). The study also revealed that CEP could downregulate Bcl-2 in colorectal cancer cells (30). Metastasis of malignant cells is also crucial in tumor-related death (31). Among matrix metalloproteinases (MMPs), MMP-9 can degrade basement membrane collagen and disrupt the barrier function of the basement membrane, allowing tumor cells to metastasize distantly (32). It has also been stated that CEP could restrain the production of MMP-9 induced by TNF-α (33). In our study, we proved that CEP had a prominent inhibitory effect on HCC cells, but did not affect normal liver cells (AML-12) in a certain concentration range. Therefore, CEP is safe in the concentration range of reducing HCC cell viability and does not damage normal hepatocytes. Besides, CEP also could suppress migration and invasion and facilitate apoptosis of HCC cells. Meanwhile, we discovered that CEP also could down-regulate CDK4, MMP-9, and Bcl-2 in HCC cells. Moreover, CEP also could reduce tumor growth of subcutaneous HCC xenografts. Thus, we further confirmed the anti-HCC activity of CEP.

HCC is characterized by excessive cell proliferation, blockage of normal apoptotic mechanisms, and metastasis, and numerous pathways have been identified to be involved (34). Among them, the JAK2/Stat3 pathway can rapidly transmit signals from extracellular to intracellular and eventually trigger biological effects (21). Thus, the JAK2/Stat3 pathway has emerged as a novel molecular target for the therapy of human tumors. Stat3 activation can be associated with downstream related factors, which can participate in regulating tumor proliferation, apoptosis, metastasis, angiogenesis, immune response, and other processes (35,36). High expression of Stat3 is usually accompanied by high expression of Cyclin D1 and C-myc in tumor cells (37). Stat3 also can suppress tumor apoptosis by downregulating p53 and Bax (38). MMP-9 expression is also positively correlated with the degree of Stat3 activation in cells (39). Studies also demonstrated that patients with high Stat3 expression in HCC have a poor prognosis; activation of Stat3 phosphorylation can accelerate HCC cell proliferation and metastasis (40,41). Stat3 expression and activation are also regulated by multiple mechanisms in the organism. It was also found that JAK2/Stat3 inhibitor (AG490) could prevent HCC cell proliferation and promote apoptosis by the JAK2/Stat3 pathway (42). In our study, we further verified that CEP could prevent HCC progression by JAK2/Stat3 pathway in vitro, and CEP also could downregulate p-JAK2 and p-Stat3 in vivo.

However, there are some shortcomings in this study. For example, this study only reveals that CEP has an anti-HCC effect and the possible mechanism is related to the JAK2/Stat3 pathway, while it cannot well simulate the syndrome type consistent with clinical manifestations; the specific mechanism by which CEP regulates JAK2/Stat3 pathway is also unclear; in vivo research is not yet complete.

## Conclusion

We demonstrated that CEP attenuates HCC progression based on the JAK2/Stat3 pathway. However, other possible pathways may also exist, which will be discussed in further experiments.

## Conflict of interest

None.

## Fundings

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