

Bigelovin inhibits hepatocellular carcinoma cell growth and metastasis by regulating the MAPT-mediated Fas/FasL pathway

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Bigelovin (BigV), as traditional Chinese medicine, has been shown to inhibit the malignant progression of hepatocellular carcinoma (HCC). This study aimed to investigate whether BigV affects the development of HCC by targeting the MAPT and Fas/FasL pathway. Human HCC cell lines HepG2 and SMMC-7721 were used for this study. Cells were treated with BigV, sh-MAPT, and MAPT. The viability, migration, and apoptosis of HCC cells were detected by CCK-8, Transwell, and flow cytometry assays, respectively. Immunofluorescence and immunoprecipitation were used to verify the relationship between MAPT and Fas. Subcutaneous xenograft tumor and tail vein-injected lung metastases mouse models were constructed for histological observation. Hematoxylin-eosin staining was used to assess lung metastases in HCC. Western blotting was used to measure the expression of migration, apoptosis, and epithelial-mesenchymal transition (EMT) marker proteins, as well as Fas/FasL pathway-related proteins. BigV treatment inhibited the proliferation, migration, and EMT of HCC cells, whereas enhanced cell apoptosis. Moreover, BigV downregulated MAPT expression. The negative effects of sh-MAPT on HCC cell proliferation, migration, and EMT were enhanced by BigV treatment. Conversely, BigV addition attenuated the positive effects of MAPT overexpression on the malignant progression of HCC. *In vivo* experiments showed that BigV and/or sh-MAPT reduced tumor growth and lung metastasis while promoting tumor cell apoptosis. Furthermore, MAPT could act with Fas and inhibit its expression. sh-MAPT upregulated the expression of Fas/FasL pathway-associated proteins, which were enhanced by BigV administration. BigV suppressed the malignant progression of HCC via activating the MAPT-mediated Fas/FasL pathway.

Key words: Bigelovin; hepatocellular carcinoma; MAPT; Fas/FasL pathway; lung metastasis

Liver cancer is a common malignancy and can be divided into two categories: primary and metastatic [1, 2]. Reportedly, approximately 41,260 new cases of liver cancer and 30,520 liver cancer-related deaths in the United States in 2022 [3]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer [4]. Currently, the treatment of HCC is mainly based on liver resection and liver transplantation, with adjuvant chemotherapy drugs. However, its therapeutic effects are not satisfactory [5, 6]. Therefore, it is an urgent issue to find a new therapeutic strategy for HCC treatment.

As a natural compound, Bigelovin (BigV), is a sesquiterpene lactone isolated from *Inula helianthus-aquatica* [7]. BigV has antioxidant, anti-inflammatory, and antiemetic properties [8, 9]. Various studies have shown that BigV inhibits the development of multiple cancer, including colorectal cancer,

multiple myeloma, and gastric cancer [10, 11]. Our previous study found that BigV suppressed the proliferation of HCC cells and induced apoptosis and autophagy [12]. However, the action mechanism of BigV against HCC has not been fully elucidated.

Microtubule-associated protein tau (MAPT) is an intrinsically disordered protein that is expressed primarily in the peripheral nervous system but is also observed in the spinal cord and skeletal muscle [13]. Abnormal aggregation of MAPT can lead to various neurological disorders, such as Alzheimer's disease and Parkinson's disease [14, 15]. Recent studies have shown a link between abnormal expression of MAPT and cancer. For example, MAPT is highly expressed in breast and lung cancer tumors and is associated with paclitaxel resistance [16, 17]. Surprisingly, using SwissTargetPrediction (<http://www.swisstargetprediction.ch>) tools,

we found that MAPT is the most probable protein target of BigV, and molecular docking analyses showed that BigV has a good binding ability with MAPT. Therefore, we guess that BigV ameliorates HCC by regulating the expression of MAPT.

FasL is a natural ligand of Fas in the body, mainly secreted by immune cells such as T cells, NK cells, and some tumor cells. The Fas/FasL pathway is an important pathway for mediating apoptosis [18]. Some studies have shown the Fas/FasL pathway is involved in the development of a variety of diseases, such as stomach cancer [19], aplastic anemia [20], and herniated lumbar discs [21]. Moreover, it is reported that regulating the Fas/FasL signaling pathway effectively promoted the apoptosis of activated hepatic stellate cells and reduced liver fibrosis [22]. Furthermore, we found that MAPT can bind to Fas by searching the STING database, so we suspected that BigV against HCC through the MAPT-mediated Fas/FasL pathway.

In this study, we used *in vivo* and *in vitro* experiments to verify whether BigV inhibits the malignant progression of HCC by regulating the MAPT-mediated Fas/FasL pathway. Our study provides a new perspective on the treatment of HCC with BigV, and MAPT may be a potential target for HCC treatment.

Materials and methods

BigV preparation. BigV (purity >99%) was purchased from MedChemExpress (Shanghai, China), and a concentration of 100 mg/ml (diluted in DMSO) was used in the study.

Cell culture and treatment. Human HCC cell lines HepG2 and SMMC-7721 (ATCC, VA, USA) were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and maintained in an incubator under 5% CO₂ at 37 °C.

HepG2 and SMMC-7721 cells were treated with 0, 5, and 10 μM of BigV for 24 h. Adenovirus vector carrying shRNAs to MAPT (sh-MAPT-1 and sh-MAPT-2), overexpression of MAPT (MAPT), and their negative control (sh-NC and vector) (GenePharma, Shanghai, China) were transfected into SMMC-7721 or HepG2 cells using Lipofectamine 3000 (Invitrogen, CA, USA).

Cell counting kit-8 assay. The viability of HepG2 and SMMC-7721 cells was measured using a Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) based on the manufacturer's instructions.

Transwell assay. Cell migration and invasion were examined using Transwell assay. For cell migration, cells (1×10⁵) were resuspended with serum-free medium and placed in the upper chamber (Corning, NY, USA). For cell invasion, the upper chamber was pre-coated with Matrigel. The complete medium was placed in the bottom chamber. After 24 h incubation, cells in the bottom chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a microscope (Zeiss, Germany).

Flow cytometry assay. The apoptosis of HepG2 and SMMC-7721 cells was detected by an Apoptosis Detection Kit (Beyotime). Shortly, 1×10⁴ cells were resuspended in binding buffer and incubated with Annexin V-FITC for 15 min and PI for 10 min at room temperature. Cell apoptosis rates were measured using flow cytometry (BD Biosciences, CA, USA) and analyzed using the FlowJo software (Tree Star, OR, USA).

Colony formation assay. HepG2 and SMMC-7721 cells (2000/well) were seeded into 6-well plates and cultured for 10 days. Colonies were fixed with formaldehyde, stained with crystal violet, and counted by ImageJ software.

Quantitative real-time PCR analysis. Total RNAs of HepG2 and SMMC-7721 cells were isolated using TRIzol reagent (Invitrogen, CA, USA) and reversely transcribed into cDNA. Quantitative real-time PCR was carried out on a 7500 real-time PCR system (Thermo Fisher Scientific, MA, USA) with SYBR green Master Mix (TaKaRa, Dalian, China). The expression of MAPT was calculated by the 2^{-ΔΔCT} method and normalized to GAPDH. The primers for qRT-PCR are as follows: (MAPT) forward, 5'-AAAGACGGGACTGGAAGCG-3' and reverse, 5'-GAATCCTGGTGGCGTTGG-3'; (GAPDH) forward, 5'-GAGAAGGCTGGGGCTCATTT-3' and reverse, 5'-AGTGATGGCATGACTGTGG-3'.

Immunofluorescence. After fixing with 4% paraformaldehyde, cells were blocked with 5% fetal bovine serum for 1 h and incubated with MAPT (1:400, ab196364, Abcam, UK) and Fas (1:500, ab126772, Abcam) antibodies at 4 °C overnight. The following day, cells were incubated with FITC/Cy3-labeled secondary antibodies (1:1000, ab6717/ab6939, Abcam) for 1 h at 25 °C. Fluorescence images were captured with a confocal microscope (Zeiss, Germany).

Mouse model construction and treatment. sh-NC and sh-MAPT transfected SMMC-7721 cells (1×10⁶) were injected into male nude mice (5–6 weeks old, 18–22 g, GemPharmatech Co. Ltd., Nanjing, China) through the tail vein to construct a lung metastasis model. The xenograft model was constructed by right-sided subcutaneous injection of 1×10⁶ SMMC-7721 cells. After tumor volume reached 50 mm³, mice were divided into four groups (n=6/group): sh-NC, sh-MAPT, BigV, and sh-MAPT+BigV. Mice in the BigV and sh-MAPT+BigV groups were given 20 mg/kg BigV intravenously every two days. Mice in the sh-NC and sh-MAPT groups were injected with an equal amount of normal saline. The size of the tumor is measured at the specified time point. After one month, the mice were euthanized, and the tumor and lung tissues were removed. The cell apoptosis and the number of lung nodules were observed using TUNEL and hematoxylin-eosin (H&E) staining according to the reported procedure [23]. All animal experiments were approved by the Ethics Committee of The First Affiliated Hospital, Zhejiang University School of Medicine.

Western blotting. Protein samples were isolated from HepG2 and SMMC-7721 cells and tumor tissues using RIPA

lysis buffer (Thermo Fisher Scientific, MD, USA), separated by gel electrophoresis, and transferred to membranes. After blocking with skim milk, the membranes were incubated overnight with primary antibodies at 4°C, followed by incubation with secondary antibody (1:2000, ab6795, Abcam) for 1 h. Finally, protein bands were visualized by ChemiDoc XRS System (Bio-Rad, CA, USA). The primary antibodies were as follows: matrix metalloproteinase (MMP)-2 (1:1000, ab205718, Abcam), MMP-9 (1:1000, ab76003, Abcam), Bax (1:1000, ab32503, Abcam), Bcl-2 (1:1000, ab32124, Abcam), E-cadherin (E-cad) (1:10000, ab76319, Abcam), N-cadherin (N-cad) (1:5000, ab76011, Abcam), MAPT (1:1000, ab196364, Abcam), Fas (1:10000, ab126772, Abcam), FasL (1:1000, ab302905, Abcam), Fas-associated protein with death domain (FADD) (1:1000, ab108601, Abcam), pro-caspase (Cas)-3 (1:1000, ab32150, Abcam), pro-Cas-8 (1:1000, ab108333, Abcam), and GAPDH (1:10000, ab181602, Abcam).

Immunohistochemistry. Paraffin-embedded tumor tissue sections were dewaxed and hydrated, and antigen was retrieved with sodium citrate solution at 80°C. Then, sections were blocked with goat serum for 15 min and incubated overnight with MAPT (1:400, ab196364, Abcam) and Ki-67 (1:200, ab16667, Abcam) antibodies at 4°C. After that, sections were incubated with secondary antibodies (1:200, ab150077, Abcam) for 1 h, followed by incubation of DAB. Finally, images were observed under a microscope (Zeiss).

Statistical analysis. Mean with standard deviation was used for exhibiting all data. Statistical analysis was performed on GraphPad8.0 with one-/two- way analysis of variance. *p*-values <0.05 were presented as significant differences.

Results

BigV inhibits the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of HCC. To investigate the effect of BigV on HCC, we examined the viability, migration, and EMT of HepG2 and SMMC-7721 cells when treated with 0, 5, and 10 μM of BigV. As shown in Figures 1A–1C, 5 and 10 μM BigV retarded the viability and migration of HepG2 and SMMC-7721 cells, whereas enhanced cell apoptosis compared with controls (*p*<0.05). Moreover, the colony number of HepG2 and SMMC-7721 cells in the BigV 5 and 10 μM group was lower than that in the control group (*p*<0.05; Figure 1D). Furthermore, the expression of migration markers (MMP-2 and MMP-9), apoptosis markers (Bax and Bcl-2), and EMT markers (E-cad and N-cad) in HepG2 and SMMC-7721 cells was detected using western blotting. And the results showed BigV dose-dependent downregulated the expression of MMP-2, MMP-9, Bcl-2, and N-cad, while upregulated Bax and E-cad expression (*p*<0.05; Figure 1E).

BigV suppresses HCC progression via targeting MAPT. To explore the action mechanism of BigV on the malignant progression of HCC. Bioinformatics analysis showed that

MAPT expression was upregulated in the primary tumor group compared to the normal group. LIHC patients with low/medium MAPT expression have a higher survival rate than LIHC patients with high MAPT expression (Figure 2A). Moreover, qRT-PCR and western blot results showed that BigV treatment reduced mRNA and protein expression of MAPT in dose-dependent manners (*p*<0.05; Figures 2B, 2C).

To confirm that BigV affects the malignant progression of HCC by targeting MAPT, the proliferation, migration, invasion, and EMT of HCC were measured when HCC cells were treated with BigV combined with sh-MAPT/MAPT. Firstly, sh-MAPT#1 and sh-MAPT#2 can effectively reduce the mRNA and protein expression of MAPT in SMMC-7721 cells compared with the sh-NC group, and sh-MAPT#2 had a better inhibitory effect, so sh-MAPT#2 was selected for subsequent experiments. Meanwhile, MAPT overexpression significantly increased mRNA and protein expression of MAPT compared to the vector group in HepG2 cells (Figures 2D, 2E). Subsequently, as shown in Figures 2F–2I, MAPT knock-down markedly reduced the viability, colony number, and migration of SMMC-7721 cells, whereas promoted cell apoptosis (*p*<0.05). The above phenomena were further strengthened by BigV addition (*p*<0.05). Overexpression of MAPT facilitated the viability, colony number, and migration of HepG2 cells as well as inhibiting cell apoptosis, which were reversed by BigV treatment (*p*<0.05). Similarly, BigV administration facilitated sh-MAPT-induced the downregulation of MMP-2, MMP-9, Bcl-2, N-cad, and the upregulation of Bax and E-cad (*p*<0.05; Figure 2J). The results of MAPT overexpression were opposite to MAPT interference.

BigV activates the MAPT-mediated Fas/FasL pathway. To verify whether BigV inhibits the development of HCC involved in MAPT-mediated Fas/FasL pathway. First, immunoprecipitation and immunofluorescence results showed that MAPT was able to interact with Fas (Figures 3A, 3B). Second, sh-MAPT transfection significantly increased the expression of Fas/FasL pathway-related proteins (Fas, FasL, FADD, pro-Cas-3 cleaved, pro-Cas-8 cleaved) compared to sh-NC transfection, which was further enhanced by BigV treatment (*p*<0.05; Figure 3C). Third, BigV administration reversed the inhibitory effect of MAPT overexpression on the expression of Fas, FasL, FADD, pro-Cas-3 cleaved, and pro-Cas-8 cleaved proteins (*p*<0.05; Figure 3D).

BigV inhibits lung metastasis by downregulating the expression of MAPT. The xenograft and the tail vein injection lung metastasis models were constructed to further verify the effect of BigV on HCC. The tumor volume in the sh-MAPT group and the BigV group was significantly smaller than that in the sh-NC group, and the BigV administration further reduced the tumor volume (*p*<0.05; Figure 4A). The expression of Ki-67 and MAPT was inhibited by sh-MAPT or BigV treatment and further inhibited by sh-MAPT+BigV treatment (Figure 4B). Simultaneously, the TUNEL assay exhibited that cell apoptosis of tumor tissues was increased by sh-MAPT or BigV treatment and further increased by

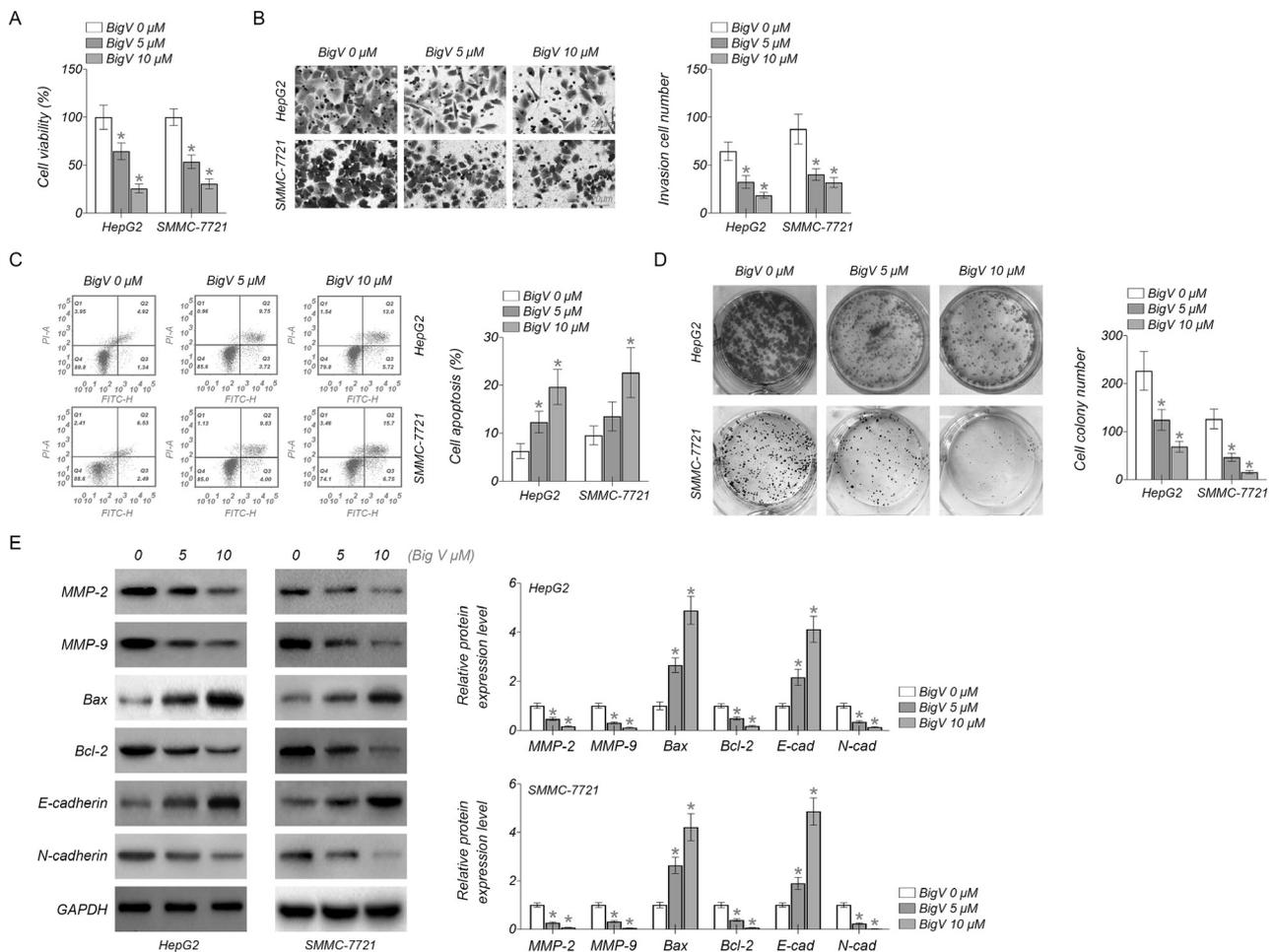


Figure 1. BigV inhibits the proliferation, migration, invasion, and EMT of HepG2 and SMMC-7721 cells were detected using Cell Counting Kit-8 (CCK-8) assay, Transwell assay, and flow cytometry assay, respectively. Scale bars: 20 μm; D) The colony number of HepG2 and SMMC-7721 cells was examined using colony formation assay; E) The expression of migration markers (MMP-2 and MMP-9), apoptosis markers (Bax and Bcl-2), and EMT markers (E-cadherin (E-cad) and N-cadherin (N-cad)) in HepG2 and SMMC-7721 cells was detected using western blotting. HepG2 and SMMC-7721 cells treated with 0, 5, and 10 μM of BigV. * $p < 0.05$ vs. BigV 0 μM

sh-MAPT+BigV treatment (Figure 4B). The combined treatment of sh-MAPT and BigV further strengthened the inhibitory effect of sh-MAPT or BigV on Fas/FasL pathway-related proteins expression ($p < 0.05$; Figure 4C). In addition, HE staining showed significant metastatic pulmonary nodules in the sh-NC group, while sh-MAPT and/or BigV treatment significantly improved this phenomenon (Figure 4D).

Discussion

HCC is the third most common cause of cancer-related mortality, characterized by high morbidity and mortality. So far, there is no effective treatment for HCC. As a traditional Chinese medicine, BigV has been shown to inhibit the growth of HCC [24]. However, its mechanism of action has not been fully elucidated. In this study, we demonstrated that

BigV could inhibit the development of HCC and lung metastasis by regulating the MAPT-mediated Fas/FasL pathway.

BigV has certain biological activities, such as antitumor, antimicrobial, antibacterial, anti-inflammatory, and other effects. At present, the research on BigV is mainly anti-tumor and reverse tumor resistance. Studies have found that BigV inhibits the growth of various tumors, such as lung, hormone-dependent prostate, breast, and epithelial ovarian cancers [25, 26]. Li et al. found that BigV reduces the proliferation and colony formation of colorectal cancer cells, whereas induces cell apoptosis [10]. Moreover, EMT is an important biological process in HCC progression, including the loss of epithelial markers (E-cad) and the increase in mesenchymal markers (N-cad) [27, 28]. Furthermore, MMP-2 and MMP-9 have been found to be involved in the migration and invasion of HCC cells [29]. In this study, we demonstrated that BigV

inhibits the proliferation, migration, and colony formation of HCC cells as well as promotes apoptosis. Meanwhile, western blotting showed that BigV downregulated the expression of MMP-2, MMP-9, Bcl-2, and N-cad, and upregulated Bax and

E-cad expression, suggesting that BigV could suppress the malignant progression of HCC.

MAPT has been shown to be highly expressed in breast and lung cancers [16, 17], which suggests that MAPT may be

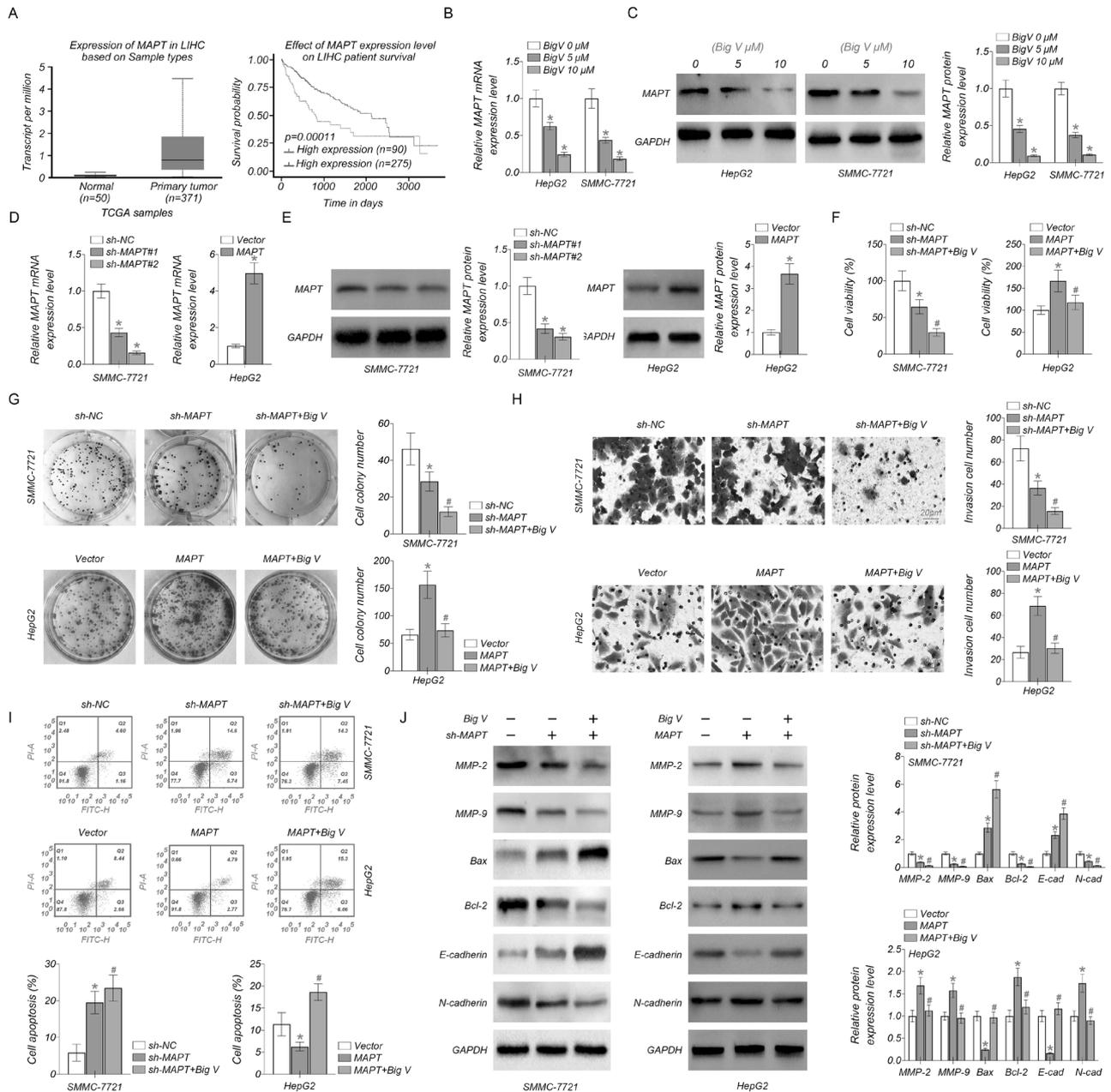


Figure 2. BigV suppresses HCC progression via targeting MAPT. A) Survival analysis of MAPT expression; B, C) mRNA and protein expression of MAPT was examined by quantitative real-time PCR (qRT-PCR) and western blotting assays. HepG2 and SMMC-7721 cells were treated with 0, 5, and 10 μM of BigV. *p<0.05 vs. BigV 0 μM; D, E) mRNA and protein expression of MAPT was examined by qRT-PCR and western blotting assays. HepG2 cells were treated with sh-NC, sh-MAPT#1, and sh-MAPT#2. SMMC-7721 cells were treated with vector and MAPT. *p<0.05 vs. sh-NC or vector; F-I) The viability, colony number, migration, and apoptosis of HepG2 and SMMC-7721 cells were detected using CCK-8, colony formation, Transwell, and flow cytometry assays respectively. Scale bars: 20 μm; J) The expression of migration markers (matrix metalloproteinase (MMP)-2 and MMP-9), apoptosis markers (Bax and Bcl-2), and EMT markers (E-cad and N-cad) in HepG2 and SMMC-7721 cells was detected using western blotting. HepG2 cells were treated with sh-NC, sh-MAPT, and sh-MAPT + BigV. SMMC-7721 cells were treated with vector, MAPT, and MAPT+BigV. *p<0.05 vs. sh-NC or vector; #p<0.05 vs. sh-MAPT or MAPT

involved in the development of cancers. Through bioinformatics analysis, we found that MAPT is highly expressed in HCC. In our study, we found that BigV could bind to MAPT and downregulate MAPT expression. Moreover, BigV treatment enhanced the effects of MAPT knockdown on the proliferation, migration, apoptosis, and EMT of HCC cells as well as reversed the affections of MAPT overexpression. All these indicate that BigV suppresses HCC progression by targeting MAPT.

Fas and its ligand, FasL, interact and constitute an important pathway for inducing cell death [18]. Fas binds to FasL and recruits FADD and cas-8 to form a death-inducing signal complex (DISC), causing apoptosis [30]. The study has shown that the increased expression of Fas and FasL can cause an increase in the expression of cas-2 and cas-8, which in turn causes apoptosis of gastric cancer cells. The study has shown that the increase in the expression of Fas and FasL can upregulate the expression of cas-2 and cas-8, which in turn causes apoptosis of gastric cancer cells [31]. In this study,

we found that MAPT could interact with Fas and inhibit Fas expression. Moreover, the BigV addition facilitated the promotion effect of sh-MAPT on the expression of Fas/FasL pathway-related proteins and weakened the inhibitory effect of MAPT on the expression of Fas/FasL pathway-related proteins. Furthermore, *in vivo* experiments exhibited that MAPT knockdown reduced tumor volume and inhibited the expression of Ki-67, MAPT, and Fas/FasL pathway-related proteins, which were further increased by sh-MAPT+BigV treatment. Additionally, HE staining showed that MAPT knockdown and/or BigV treatment significantly reduced the number of lung metastases. The above results confirm that BigV activates the Fas/FasL pathway by downregulating the expression of MAPT, thereby promoting apoptosis and thereby inhibiting the progression of liver cancer.

In conclusion, this study demonstrated that BigV inhibits HCC progression by activating the MAPT-mediated Fas/FasL pathway. Our research provides a theoretical basis for the treatment of HCC by BigV.

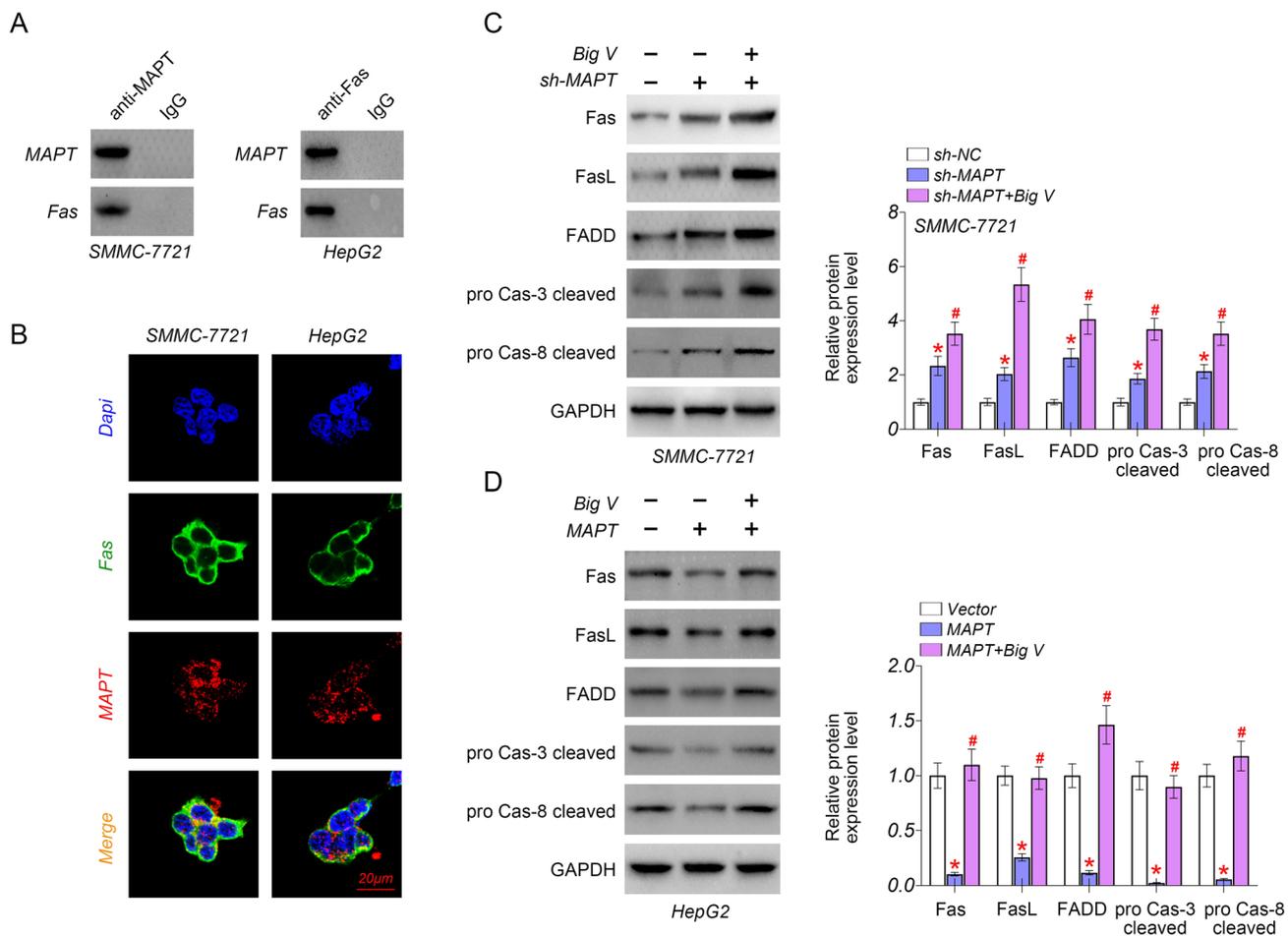


Figure 3. BigV activates the MAPT-mediated Fas/FasL pathway. **A)** Immunoprecipitation analysis of MAPT and Fas; **B)** Immunofluorescence staining analysis of MAPT and Fas. Scale bars: 20 µm; **C, D)** Western blotting was used to measure the expression of Fas/FasL pathway-related proteins (Fas, FasL, Fas-associated protein with death domain (FADD), pro-caspase (Cas)-3 cleaved, and pro-Cas-8 cleaved) in HepG2 and SMMC-7721 cells. HepG2 cells were treated with sh-NC, sh-MAPT, and sh-MAPT + BigV. SMMC-7721 cells were treated with vector, MAPT, and MAPT+BigV. * $p < 0.05$ vs. sh-NC or vector; # $p < 0.05$ vs. sh-MAPT or MAPT

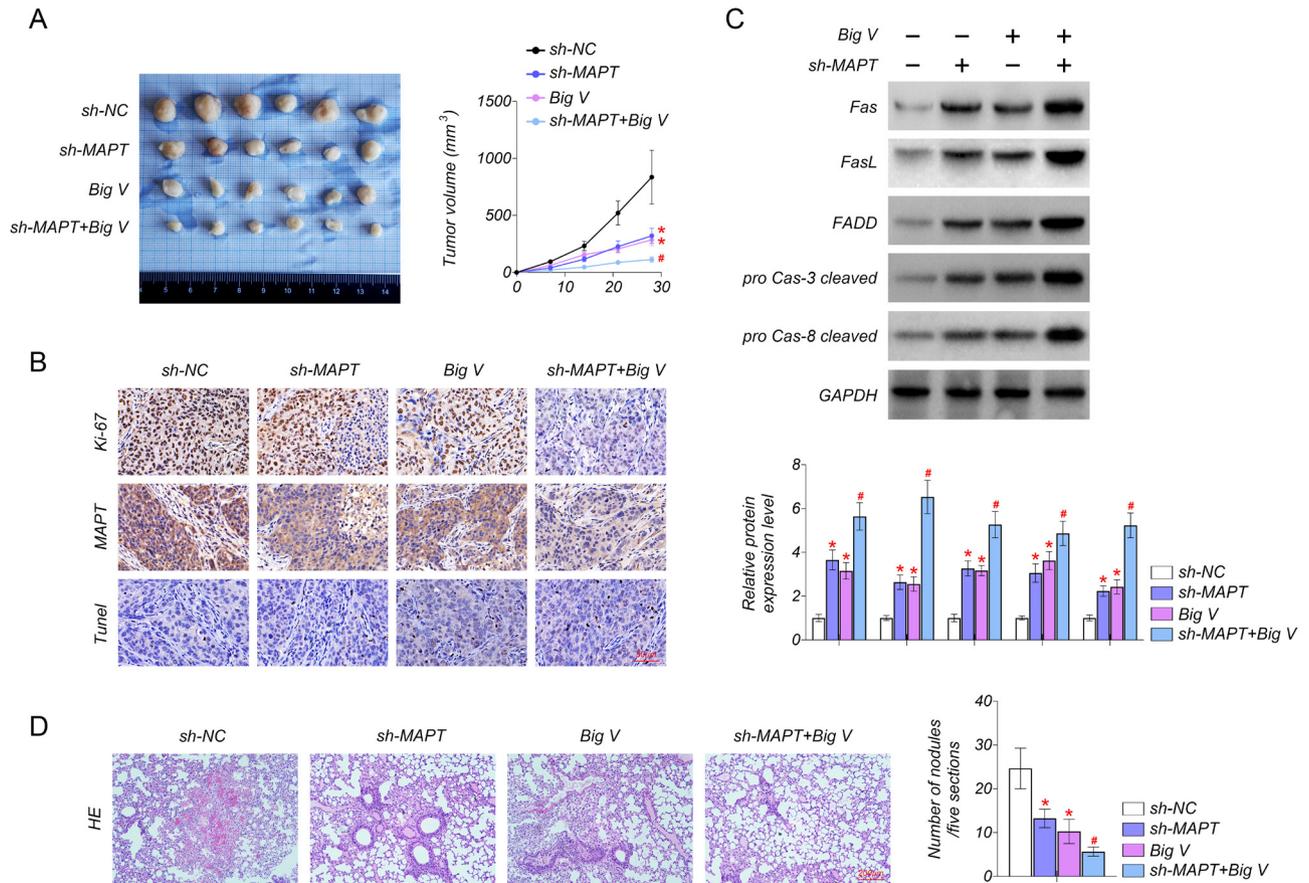


Figure 4. BigV inhibits lung metastasis by downregulating the expression of MAPT. **A)** The volume of the tumors was detected; **B)** Ki-67 and MAPT expression as well as apoptosis of tumor tissues were determined via immunohistochemistry and TUNEL assays. Scale bars: 20 μ m; **C)** The expression of Fas/FasL pathway-related proteins were detected using western blotting; **D)** The number of metastatic nodules in lung tissue was observed by hematoxylin&eosin (H&E) staining. Scale bars: 20 μ m. * p <0.05 vs. sh-NC; # p <0.05 vs. sh-MAPT

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