

# CASK Silence Enhances Chemosensitivity of Hepatocellular Carcinoma Through activating Apoptosis, Inducing JNK/c-Jun-Mediated Autophagic Cell Death and Decreasing ABCG2

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## Research

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# Abstract

**Background:** Advanced hepatocellular carcinoma (HCC) patients usually fail to be treated because of drug resistance, including sorafenib.

**Methods:** The expression and prognostic role of calcium/calmodulin-dependent serine protein kinase (CASK) in HCC were assessed by combination of bioinformatic analysis and experimental validation. The effects of CASK in regulating proliferation, apoptosis and drug resistance of HCC cells *in vitro* and *in vivo* were investigated using gain- or loss-of-function strategies by performing lots of specific methods including Cell Counting kit-8 (CCK8), colony formation assay, flow cytometry, transmission electron microscopy, immunofluorescent confocal laser microscopy and tumor xenograft experiments, immunohistochemistry staining. Moreover, the underlying molecular mechanisms responsible for CASK's functions in HCC were also explored.

**Results:** Currently, we discovered that CASK was positively associated with sorafenib resistance of HCC *in vitro* and *in vivo*, and was significantly related with poor prognosis in HCC. Moreover, inhibition of CASK can increase the effect of sorafenib partially by promoting apoptosis and autophagy, while CASK overexpression presented the opposite results. Besides, all the pan-caspase inhibitor Z-VAD-FMK, autophagy inhibitor 3-Methyladenine (3-MA) and small interfering RNA (siRNA) of LC3B reversed CASK knockout-induced effects with sorafenib treatment, suggesting that both apoptosis and autophagy were involved in CASK-mediated above functions and autophagy played a pro-death role in this research. Intriguingly, similar results were observed *in vivo*. In molecular level, CASK knockout activated the c-Jun N-terminal kinase (JNK) pathway, and treatment with JNK inhibitor SP600125 or transiently transfected with si-JNK significantly attenuated CASK knockout-mediated autophagic cell death. Besides, knockout of CASK dramatically inhibited the expression of ATP binding cassette subfamily G member 2 (ABCG2) and reversed of multidrug-resistance (MDR) of HCC.

**Conclusions:** Collectively, all these results together indicated that CASK might be a promising biomarker for HCC patients and a potential therapeutic target for relieving drug resistance of HCC.

## Background

Hepatocellular carcinoma, approximately 750,000 new cases occurred per year, is the sixth most frequently neoplasm and ranks the third leading cause of cancer-related deaths worldwide [1–3]. Currently, 30% HCC patients present with advanced stages, while treatment options are limited, accompanied with dismal survival rate [4]. The molecular targeted agent, sorafenib, remains one of the first-line systemic drugs of advanced HCC patients with overall survival benefit [5, 6]. However, sorafenib resistance limits efficacy of this treatment in HCC cases. Although some conditions or pathways that lead to sorafenib resistance including AKT activation, hypoxic environment, Epithelial-mesenchymal transition (EMT), cancer stem cells/tumor-initiating cells, Epidermal growth factor receptor (EGFR) activation, c-Jun activation and autophagy have been reported [7], more efforts should to be put to further elucidate the

complicated sorafenib resistance mechanism, thereby improving the outcome of sorafenib treatment in HCC patients. It's necessary to elucidate the sorafenib resistance mechanism and improve the treatment outcome.

Calcium/calmodulin-dependent serine protein kinase (CASK), a scaffold protein from membrane-associated guanylate kinase (MAGUK) protein family, plays significant roles in the neuronal system [8, 9]. However, the researches about this gene in cancer are still limited. To date, some previous studies have reported that CASK closely links to tumor development. For example, Wei, J. L. *et al.* found that high expression level of CASK was associated with poor prognosis and progression of colorectal cancer [10]; Zhou, X. *et al.* revealed that decreased the expression of miR-203 promoted the proliferation and invasion of gastric cancer through targeting CASK [11]. These observations suggest that CASK may play an important role in cancer, while the research of CASK in HCC is poorly explored but necessary to be further studied.

Apoptosis, or programmed cell death, is a common mechanism that sensitizes cancer cells to chemotherapy agents and is regulated by many apoptosis-related proteins and pathways [12]. Besides, autophagy is a self-degradative cellular process, and it plays a key role in the development and progression of tumor, including drug resistance [13]. Chemotherapy agents, such as sorafenib, Fluorouracil (5-FU), may induce autophagy, but the role of autophagy is indistinct. On the one hand, autophagy can lead to chemoresistance through a cell survival mechanism [14–16]. On the other hand, overactivated autophagy may lead to autophagic cell death or non-apoptotic form of programmed cell death and relieve drug resistance [17–21]. Interestingly, mounting evidence shows that apoptosis and autophagy closely cross talk with each other [22, 23]. Therefore, targeting apoptosis or autophagy may provide a potential and promising therapeutic strategy to overcome chemoresistance and relieve progression for patients with cancer.

However, during the therapy, many patients gradually develop resistance to not just one drug, but also to many different drugs. This phenomenon is also called as multidrug resistance and it will seriously affect the therapy efficiency [24]. ATP-binding cassette (ABC) transporters are abundantly expressed in various human tissues including liver and play a crucial role in absorption, distribution, and excretion of drugs, and also be reported to be closely related with drug resistance [25, 26]. ATP binding cassette subfamily G member 2 (ABCG2), also known as BCRP, has been indicated to be associated with MDR in many tumors [27–29]. Hence, ABC transporters, like ABCG2, may be significant switch molecules for the effectiveness of anti-HCC chemotherapeutic agents during the treatment.

Our data for the first time suggest that high expression of CASK was positivity associated with multi-drug resistance of HCC cells and poor prognosis of HCC patients. Furthermore, we confirmed that depletion of CASK augmented the chemosensitivity of HCC by activating apoptosis, enhanced JNK/c-Jun pathway-mediated autophagic cell death and reduced ABCG2-mediated drug efflux. Overall, our study demonstrate that CASK will hopefully become a potential biomarker for HCC patients and provides a brand-new strategy for HCC therapy, and it is worth in-depth study in the further.

# Materials And Methods

## Clinical samples

60 HCC tissues and paired normal tissues were obtained from the First Affiliated Hospital of Zhejiang University (diagnosed from 2017 to 2018). All patients signed the informed consent. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhejiang University. The diagnosis of patient was confirmed by histopathological examination.

## Cell culture and siRNA transfection

SMMC-7721 were cultured in RPMI-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA). And SMMC-7721-sora were cultured in RPMI-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA) and 2000 ng/ml sorafenib. All cell lines were maintained at 37°C and 5% CO<sub>2</sub> in incubator.

siRNA for CASK, LC3B and JNK were synthesized by RiboBio Co., Ltd (Guangzhou, China) (**Table S1**). HCC cells were seeded in 6 or 96-well plates, transfected after 12 hours according to the manufacturer's protocol and treated with drug after transfection for 72 h.

## Plasmids construction and transfection

CASK expression was knocked out using CASK sgRNA CRISPR/Cas9 system (target sequences: 5'-CGACGACGACGTGCTGTTTCG-3') and stable expression cell lines were selected using puromycin. pcDNA3.1 and pcDNA-CASK plasmids were designed by Replibio Co., Ltd (Hangzhou, China) and stable expression cell lines were selected using G418. All these transfections were performed according to the manufacturer's instruction.

## Cell viability assay and colony formation assay

Cell viability assays were performed by the CCK8 assays. Cells were plated into 96-well plates with 3000 cells per well. After 12 hours, cells were transfected. Next, cells were exposed with drug treatment and cultured for 72h. Subsequently, 10 ul of CCK8 was added to each well for 4 h, then the OD value was measured at 450 nm. HCC cells were seeded into 6-well plates for colony formation assay (1000 cells/well). After maintained at 37°C and 5% CO<sub>2</sub> in incubator for 2 weeks, the cells were stained with Wright's-Giemsa stain according to the manufacturer's instructions. The number of colonies more than 50 cells/colony were counted.

## Flow cytometry analysis

The cells were divided into four groups according to the experimental requirements (si-NC, si-CASK-3, si-NC + sorafenib, si-CASK-3 + sorafenib). At first,  $20 \times 10^4$  si-NC or si-CASK-3 transfected cells were plated into 6-well plates, then cultured with or without sorafenib for 72h according to experiment groups (SMMC-

7721-sora:8  $\mu\text{M}$  sorafenib, SMMC-7721: 2  $\mu\text{M}$  sorafenib). An Annexin V-FITC/PI apoptosis kit (Multi Sciences, Biotech, China) was used to detect cell apoptosis based on the manufacturer's protocol. Data analyses were performed with BD FACSCalibur™ flow cytometry system.

## RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues and cells using RNAiso plus Reagent (TaKaRa, Kusatsu, Japan) and reverse transcribed to cDNA using PrimeScript RT Reagent Kit (TaKaRa, RR0037A). cDNA was analyzed by qRT-PCR using SYBR Premix Ex Taq (TaKaRa, RR420A). GAPDH was used as an endogenous control. The relative expression values were calculated using  $2^{-\Delta\Delta\text{CT}}$  method. Gene-specific primers were listed in **Table S1**.

## Chemotherapeutic agents and antibodies

The antibodies including Anti-GAPDH, Anti-CASK, Anti-PARP, Anti-Cleaved PARP, Caspase-7, Anti-LC3B, SQSTM1/p62, Beclin-1, Anti-JNK1 + JNK2 + JNK3, Anti-phospho-JNK, Anti-c-Jun, Anti-phospho-c-Jun, Anti-MRP3, Anti-ABCG2 were listed in Table S2. JNK inhibitor SP600125 (MB5595), 3-Methyladen (3-MA, MB5063), Z-VAD-FMK (MB3313), sorafenib (MB1666), doxorubicin (MB1087), daunorubicin (MB1074), decitabine (5-Aza, MB1075), G418 (MB1733), puromycin (MB2005) were purchased from Dalian Meilun Biotechnology.

## Western blot analysis

Total proteins were lysed with RIPA buffer containing with protease inhibitor and phosphatase inhibitor on ice and centrifuged at 14000 rpm to remove the debris. A BCA protein assay kit (Beyotime Biotec, China) was used to measure concentrations of proteins. Equal amount of protein sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 hour, and incubated with primary antibodies overnight at 4°C. After that, the membrane was incubated with secondary antibody for 1h at room temperature. The results were visualized and analyzed by ECL detection solution (Thermo Scientific™) and Image Lab software (Bio-Rad), respectively.

## Transmission electron microscopy (TEM)

About  $5 \times 10^6$  si-NC or si-CASK-3 transfected cells treated with or without 12.5  $\mu\text{M}$  sorafenib were collected and fixed in 2.5% glutaraldehyde at least for 4h. All the samples were treated with 1% osmium tetroxide and dehydrated in graded concentrations of ethanol and acetone, finally samples were embedded in Durcupan resin. Ultrathin sections (70 nm) were examined under a JEM-1230 electron microscope (JEOL, Japan) at 80 kV.

## Tumor xenograft experiments

All the experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Male nude athymic mice (3–5 weeks old) were purchased from SLAC Laboratory Animal CO., Ltd (Shanghai, China). A stable CASK knockout cell line of SMMC-7721-sora was established (SMMC-

7721-sora sg-CASK). In order to determine the effect of CASK knockout on sorafenib resistance in vivo, a total of  $5 \times 10^6$  cells were injected into the right flank of each immune-deficient nude mice (control group or sg-CASK group, N = 10 per group). 6 days later, all of the animals in each group were divided into another two groups (Dimethyl sulfoxide (DMSO) treated or sorafenib treated, 10mg/kg) and administered every 3 days. Additionally, to explore the role of CASK regulates autophagy and augments sorafenib sensitivity in vivo, a total of  $5 \times 10^6$  cells were injected into the immune-deficient nude mice. 6 days later, all of the animals were divided into four groups (no treated or 3-MA treated or sorafenib treated or combination of 3-MA and sorafenib treated, 3-MA: 20mg/kg, sorafenib: 10mg/kg, N = 4 per group) and administered every 3 days. Mice body weight and tumor volume ( $1/2 \times \text{length} \times \text{width}^2$ ) were measured every 3 days, continue to day 24. Besides, after the animals were terminated, tumor tissues were separated and weighted.

## **Immunofluorescent confocal laser microscopy**

SMMC-7721-sora control cells or SMMC-7721-sora sg-CASK cells were grown on coverslips and transfected with a GFP-LC3 plasmid overnight and pretreated with or without 20  $\mu\text{M}$  SP600125 for 2h. Then, cells were treated with 12.5  $\mu\text{M}$  sorafenib or not treated for another 36h. After that, cells were fixed with 4% paraformaldehyde for 15 min and stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Images were taken under confocal fluorescence microscopy (Nikon AIR confocal microscope).

## **Immunohistochemistry (IHC) staining**

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining were performed to gain the expression of CASK, LC3B, p-JNK and p-c-Jun in 4- $\mu\text{M}$ -thick paraffin-embedded sections from tumor xenografts. Slides were examined using an optical microscope (Olympus). At least 7 randomly selected 40x fields of Ki67 staining were visualized and the percentage of positive nuclei were quantified using Image J software (NIH, Bethesda, USA).

## **Statistics**

Experimental data were performed using GraphPad Prism software (version 7.0.3) and expressed as mean  $\pm$  standard deviation (SD) of at least three independent experiments. Differences between two groups were analyzed using unpaired Student's *t*-test and p value less than 0.05 was indicated statistically significant.

## **Results**

### **Hypomethylation-associated upregulation of CASK expression in HCC was positively correlated with poor prognosis**

To explore the potential function of CASK in HCC, we first analyzed the expression of CASK through bioinformatic analysis and experimental validation. The results showed that the messenger RNA (mRNA)

expression of CASK was significantly upregulated in HCC tissues compared with normal liver tissues in The Cancer Genome Atlas (TCGA) (<https://cancergenome.nih.gov/>) (Fig. 1A), and IHC staining performed by the human protein atlas (HPA) database (<https://www.proteinatlas.org/>) showed strongly positive CASK staining in HCC cancer tissue, but weakly positive staining in normal tissue (Fig. 1B). Receiver operating characteristic (ROC) curve analysis indicated that the expression of CASK could effectively distinguish HCC from normal tissues (Fig. 1C). And upregulation of CASK expression was associated with advanced stage in HCC (Fig. 1D). Furthermore, we compared the expression of CASK in 60 paired HCC samples and corresponding normal samples and found that CASK was markedly upregulated in HCC cancer samples (Fig. 1E). Besides, the Kaplan-Meier analysis (<http://kmplot.com/analysis/>) indicated that HCC patients with high-CASK expression demonstrated poor overall survival rate, and consistent result was observed in HCC patients treated with sorafenib (Fig. 1F and Fig. 1G). Promoter DNA methylation is closely related with gene expression [30, 31]. Thus, a possible link between promoter hypomethylation and upregulation of CASK expression in HCC was investigated. According to the analysis from UALCAN database (<http://ualcan.path.uab.edu/>), we found that promoter methylation level of CASK was remarkably decreased in HCC tumor tissues compared with normal tissues (Fig. 1H). Moreover, SMMC-7721 cells treated with 5  $\mu$ M or 10  $\mu$ M demethylation agent, 5-AZA, significantly increased CASK mRNA levels (Fig. 1I). Collectively, all the data suggested that promoter hypomethylation-associated CASK upregulation in human HCC was positively related with sorafenib resistance and poor prognosis.

Knockdown of CASK inhibited HCC proliferation, promoted HCC apoptosis and enhanced the sorafenib sensitivity in vitro

SMMC-7721-sora and SMMC-7721 cells were transfected with specific siRNAs targeting CASK (si-CASK-1, si-CASK-2, si-CASK-3) and negative control si-NC, the qRT-PCR assay indicated that si-CASK-3 with the best effect in inhibiting CASK expression (**Figure S1A** and **Figure S1B**). Additionally, the western blotting analyses were further performed to verify the effect of si-CASK-3 in SMMC-7721-sora and SMMC-7721 cells (Fig. 2A). And CCK8 assay showed that after decreasing expression of CASK, the sorafenib treatment was significantly sensitive in various concentrations in SMMC-7721-sora and SMMC-7721 cells (Fig. 2B and Fig. 2C). Besides, overexpression of CASK showed opposite consequences in SMMC-7721-sora and SMMC-7721 cells (**Figure S1C** and **Figure S1D**). For colony information assay, HCC cells were treated with si-CASK-3 or si-NC for 12 h, then cultured with sorafenib (sorafenib concentrations: 8  $\mu$ M for SMMC-7721-sora, 2  $\mu$ M for SMMC-7721) for 14 days. The assay further indicated that downregulation of CASK significantly decreased clonogenicity of SMMC-7721-sora and SMMC-7721 cells, and the effect was more pronounced when co-treated with sorafenib (Fig. 2D and Fig. 2E). Furthermore, CASK upregulation combined with sorafenib treated notably increased clonogenicity of SMMC-7721-sora and SMMC-7721 cells compared with control groups (**Figure S1E** and **Figure S1F**). To further define the mechanism of CASK-induced sorafenib chemoresistance, cells transfected with si-CASK-3 or si-NC cultured with varying concentrations of sorafenib or DMSO were analyzed for the apoptotic marker changes by the flow cytometric analyses. Markedly, a significant increase in apoptosis was observed in CASK-knockdown cells compared with control cells, and the effect was more noticeable when combined

with sorafenib (Fig. 2F and Fig. 2G). To further confirm these consequences, cleaved PARP and cleaved caspase-7, two markers of apoptosis, were monitored by western blotting analysis. As expected, the levels of cleaved PARP and cleaved caspase-7 were increased upon inhibition of CASK, with or without sorafenib treatment (Fig. 2H and Fig. 2I). In contrast, overexpression of CASK in SMMC-7721-sora and SMMC-7721 cells had the opposite effects, leading to inhibit sorafenib-induced apoptosis (**Figure S1G** and **Figure S1H**).

## **Silence of CASK increased autophagy of HCC cells and activated the JNK/c-Jun signaling pathway**

In order to further discover the potential molecular mechanism underlying the increased drug sensitivity induced by decreased CASK expression, we performed the gene set enrichment analysis (GSEA) using the data from TCGA. We interestingly found that CASK expression was closely related with regulation of autophagy (Fig. 3A). Since autophagy is a significant regulatory process in maintaining the cellular homeostasis, and plays a dual role in chemoresistance, we hypothesized that regulation of autophagy may participated in CASK-mediated sorafenib resistance. To test this, we investigated the levels of LC3B, p62 and Beclin-1 in SMMC-7721-sora and SMMC-7721 cells. Western blotting results showed that CASK knockdown significantly increased the expression of autophagic marker LC3B-II and Beclin-1, and decreased SQSTM1/p62 expression, with or without sorafenib treatment, whereas CASK overexpression decreased the expression of those proteins when treated with sorafenib (Fig. 3B and **Figure S2A**). The TEM results revealed that CASK-knockdown cells contained more autophagosomes in the cytoplasm compared with control cells, with or without sorafenib treated (Fig. 3C).

Increasing studies have confirmed that JNK pathway activation plays a pivotal role in regulating autophagy, and is closely related with chemoresistance and tumor progression [32–34]. To further investigate the mechanism of CASK knockdown-induced autophagy, the effect of CASK knockdown on the JNK pathway was detected. Western blotting analysis indicated that CASK knockout indeed led to the increase of the protein levels of phosphorylated-JNK (p-JNK) and phosphorylated-c-Jun (p-c-Jun) in SMMC-7721-sora and SMMC-7721 cells with or without sorafenib treated (Fig. 3D).

## **Knockout of CASK inhibited HCC cell tumorigenesis and increased the effect of sorafenib in vivo**

SMMC-7721-sora and SMMC-7721 cells with stable knockout of CASK using CRISPR/Cas9 were screened out for the next research, and western blotting assay was performed to validate the effective knockout of CASK (Fig. 4A and Fig. 4B). Then, SMMC-7721-sora (control cells) and SMMC-7721-sora sg-CASK cells were selected to conduct *in vivo* analysis. As shown in the diagram (Fig. 4C), a total of  $5 \times 10^6$  control cells or sg-CASK cells in 100 ul PBS were injected into the mice, respectively. 6 days later, each group mice were further injected with 10 mg/kg of sorafenib every 3 days or DMSO every 3 days, and tumor volumes were measured. We found that the tumors derived from control cells grew evidently faster than those from sg-CASK cells, and the difference was more obvious when treated with sorafenib

(Fig. 4D). Likewise, tumor weight of xenografts derived from CASK suppression demonstrated a superior response to sorafenib compared to controls (Fig. 4E). Consistently, the mean volume of tumors in CASK knockout groups showed markedly smaller than in controls groups, especially in combination of sorafenib treated (Fig. 4F). H&E staining and Ki67 staining further indicated that knockout of CASK significantly inhibited proliferation (Fig. 4G). Together, these results suggested that depletion of CASK inhibited HCC cell tumorigenesis, increased apoptosis and enhanced the therapeutic effect of sorafenib *in vivo*.

## **CASK depletion modulated autophagic cell death-mediated sorafenib sensitization through activating JNK/c-Jun signaling pathway**

In the next step, we want to figure out whether CASK depletion-triggered autophagy showed a pro-survival or pro-death role, and whether it is mediated by JNK/c-Jun signaling pathway. Hence, a pan-caspase inhibitor (Z-VAD-FMK) was applied to CASK knockout treatment at first. And the CCK-8 assay indicated that Z-VAD-FMK treatment partially reversed CASK knockout-induced cell death (Fig. 5A). It suggested that CASK was involved in the regulation of sorafenib resistance by regulating apoptosis, but non-apoptotic form of cell death might exist. Next, an autophagy inhibitor (3-MA) and the siRNA of LC3B were applied to inhibit autophagy. Western blotting was performed to detected the effect of 3-MA and si-LC3B (**Figure S2B**). The CCK-8 assay indicated that 3-MA and si-LC3B treatment noticeably suppressed CASK knockout-induced cell death (Fig. 5B and Fig. 5C). Then, to determine whether CASK knockout activated autophagic cell death to sensitize HCC cells of sorafenib *in vivo*, xenograft tumor models of SMMC-7721-sora sg-CASK cells were generated. A total of 16 male nude athymic mice were randomly divided into 4 groups, including the control group, 3-MA group, sorafenib group, and sorafenib plus 3-MA group. As shown in Fig. 5D and Fig. 5E, the tumor size of 3-MA group was significantly larger than that without 3-MA treated group, especially in combination with sorafenib treated. Taken together, all these data support that inhibition of autophagy attenuates CASK knockout-induced cell death.

To determine whether CASK knockout-induced autophagy was dependent on JNK pathway activation, we specifically inhibited JNK signaling pathway pretreated with JNK-specific inhibitor SP600125, and CCK8 assay showed that inhibition of JNK/c-Jun signaling pathway with SP600125 attenuated the cytotoxicity activity of sorafenib in CASK knockout cells (Fig. 5F). Western blotting data further showed that SP600125-mediated inhibition of JNK significantly decreased the expression levels of JNK phosphorylation and LC3B-II in CASK knockout cells (Fig. 5G). Similar consequence was occurred when JNK/c-Jun pathway was inhibited by siRNA of JNK (Fig. 5H and Fig. 5I). In accordance with these results, laser confocal images indicated that the number of LC3 positive puncta in CASK knockout cells were obviously increased than in control cells, and SP600125 pre-treatment significantly inhibited CASK knockout-induced LC3 positive puncta numbers (Fig. 5J). In addition, immunohistochemistry analyses of mice tumor tissues also showed that LC3B-II expression, p-JNK expression and p-c-Jun expression were

higher in sg-CASK tissues than that in the control tissues with or without sorafenib treated (Figure S2C). These data illustrated that JNK/c-Jun signaling pathway was involved in CASK-mediated autophagy.

## **ABCG2 was involved in CASK-regulated multi-drug resistance of HCC cells**

In addition, we found that the drug-sensitizing effect could be observed in other anticancer drugs, including doxorubicin and daunorubicin (Fig. 6A and Fig. 6B). By pumping the drugs outside from cancer cells and attenuate the potency of chemotherapeutics, ATP-binding cassette (ABC) transporters superfamily often involves in chemoresistance [35–37]. At first, we studied the expression changes of those drug efflux pump proteins closely related with drug resistance in HCC. qRT-PCR indicated that the mRNA expressions of ATP binding cassette subfamily C member 3 (ABCC3) and ABCG2 were significantly decreased when knockdown of CASK (Fig. 6C and Fig. 6D), and positive correlation were found between CASK and ABCC3 or ABCG2 from GEPIA (<http://gepia.cancer-pku.cn/>) and starbase (<http://starbase.sysu.edu.cn>) database (Fig. 6E and Fig. 6F). While knockout of CASK only significantly downregulated the protein level of ABCG2 when treated with sorafenib, but not MRP3 (Fig. 6G and Fig. 6H). All results confirmed that ABCG2 was involved in CASK-regulated multi-drug resistance of HCC cells.

## **Discussion**

Chemoresistance is one of the major obstacles to improve the life quality and survival time of HCC patients. Elucidation the mechanism of drug resistance will help to identify potential and effective therapeutic targets to reverse drug resistance of HCC. This study illustrated that CASK was important for the sorafenib resistance of HCC cells *in vitro* and *in vivo* and further explored the underlying mechanism of CASK in HCC pathogenesis and progression.

Firstly, we found that CASK expression was significantly upregulated in HCC and was closely related with poor prognosis for HCC patients, which was regulated by promoter hypomethylation. More importantly, our data first showed that CASK depletion-mediated sorafenib sensitization *in vitro* and *in vivo* mainly through increasing apoptosis, autophagy and decreasing the expression of ABCG2. It's well to known that the cytotoxic effect of chemotherapeutic drugs relies on their ability to induce apoptosis, also known as programmed cell death. Importantly, evading apoptosis is a common and key characteristic of cancer cells and is responsible for chemoresistance [38, 39]. In the current study, the experimental data revealed that CASK downregulation increased HCC cell apoptosis through enhancing cleaved PARP and cleaved caspase 7 activation. Although apoptosis is the most widely studied programmed cell death, recent analyses have highlighted the significance of additional forms of cell death, like autophagic cell death [40–42]. In this research, we found that CASK knockout-induced autophagy per se enhanced its cell death effect. Expect apoptosis and autophagy, necrosis is another major mechanism explore for mammalian cell death, and we will demonstrate the relationship between CASK and necrosis in our future study.

Previous researches have indicated that JNK/c-Jun signaling pathway that belongs to mitogen-activated protein kinase (MAPK) pathway, has vital function in regulating autophagic cell death. For example, Bai, Y. *et al* have reported that PDIA6 knockdown suppressed NSCLC cell proliferation and increased cisplatin-induced autophagic cell death *via* interacting with MAP4K1 to activate the JNK/c-Jun signaling pathway [43]; Hu, L. *et al* have proved that SNX-2112, the Hsp90 inhibitor, enhanced TRAIL-induced apoptosis and autophagy of cervical cancer cells through activating the ROS-regulated JNK-p53-autophagy-DR5 pathway [32]; Zhu, Q. *et al* indicated that irinotecan (IRI) stimulated the reactive oxygen species (ROS)-related JNK- and p38-MAPK signaling pathways to increase autophagy-dependent apoptosis and inhibit growth of gastric cancer cells [44]. Therefore, the active status of the JNK/c-Jun signaling pathway was detected under CASK knockdown condition in the present study. As expected, we indeed observed that phosphorylation of JNK/c-Jun was significantly increased when CASK knockdown with the presence or absence of sorafenib treatment. In addition, inhibition of JNK *via* SP600125 or siRNA markedly suppressed sorafenib induced-autophagy in CASK knockdown SMMC-7721-sora cells. The consequences indicated that the JNK/c-Jun pathway was partially responsible for CASK knockout-medicated autophagic cell death.

An active efflux mechanism is one of the main reasons for MDR in cancer. Recently, mounting studies have highlighted the critical role of ABCG2 in mediating multidrug resistance of HCC cells [45, 46]. Our study first revealed that ABCG2 was involved in CASK-regulated MDR, providing a novel insight into how CASK regulated sorafenib resistance in HCC. Moreover, it's surprising to found that the mRNA and protein expression of CASK in parental cells and sorafenib-resistance cells were on the contrary (data not show), more efforts need to be put to explore this phenomenon. Besides, in order to further improve the clinical value of our experimental study, the clinical significance of CASK detection in sorafenib sensitivity or resistance HCC tissues in predicting the chemotherapy response or survival rate is also worth study in the future.

In conclusion, our study demonstrated that hypomethylation-induced upregulation of CASK in HCC is associated with poor prognosis for HCC patients. Furthermore, activation of apoptosis and JNK/c-Jun signaling pathway mediated autophagic cell death, and downregulation of ABCG2 produced by CASK downregulation, which reinforces sorafenib's effect in HCC cells (Fig. 7). Thus, CASK may serve as a potential novel prognostic indicator in HCC, and targeting CASK may be a promising strategy for HCC patients, especially for sorafenib resistant HCC patients.

## Conclusions

In summary, our analysis for the first time showed that hypomethylation-mediated high expression of CASK in hepatocellular carcinoma is associated with poor prognosis for hepatocellular carcinoma patients, and depletion of CASK enhances the sorafenib sensitivity *in vitro* and *in vivo* through activating apoptosis and autophagic cell death and decreasing ABCG2 expression.

## Abbreviations

HCC: hepatocellular carcinoma; CASK: calcium/calmodulin-dependent serine protein kinase; CCK8: cell counting kit-8; 3-MA: 3-Methyladenine; 5-Aza: decitabine; MDR: multidrug-resistance; EMT: Epithelial-mesenchymal transition; EGFR: Epidermal growth factor receptor; MAGUK: membrane-associated guanylate kinase; 5-FU: Fluorouracil; ABC: ATP-binding cassette; ABCG2: ATP binding cassette subfamily G member 2; ABCC3: ATP binding cassette subfamily C member 3; TEM: Transmission electron microscopy; DMSO: Dimethyl sulfoxide; DAPI: 4',6-diamidino-2-phenylindole; H&E: Hematoxylin and eosin; IHC: immunohistochemistry; mRNA: messenger RNA; TCGA: The Cancer Genome Atlas; HPA: human protein atlas; ROC: Receiver operating characteristic; siRNA: small interfering RNA; qRT-PCR: quantitative real-time PCR; GSEA: gene set enrichment analysis; JNK: c-Jun N-terminal kinase; p-JNK: phosphorylated-JNK; p-c-Jun: phosphorylated-c-Jun; MAPK: mitogen-activated protein kinase; ROS: reactive oxygen species

## **Declarations**

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### **Availability of data and materials**

Not applicable

### **Authors' Contributions**

Designed the experiments: Bisha Ding, Zhijun Dai, Weimin Fan and Weiyang Lou. Performed the experiments: Bisha Ding, Chang Bao, Luqi Jin. Write the manuscript and analyzed the data: Bisha Ding, Liang Xu. Discussion, supervised all the work and reviewed this manuscript: Weiyang Lou, Weimin Fan and Zhijun Dai. All authors have read and approved the final version of the manuscript.

### **Ethics approval and consent to participate**

Not applicable

### **Patient consent for publication**

Not applicable

### **Competing interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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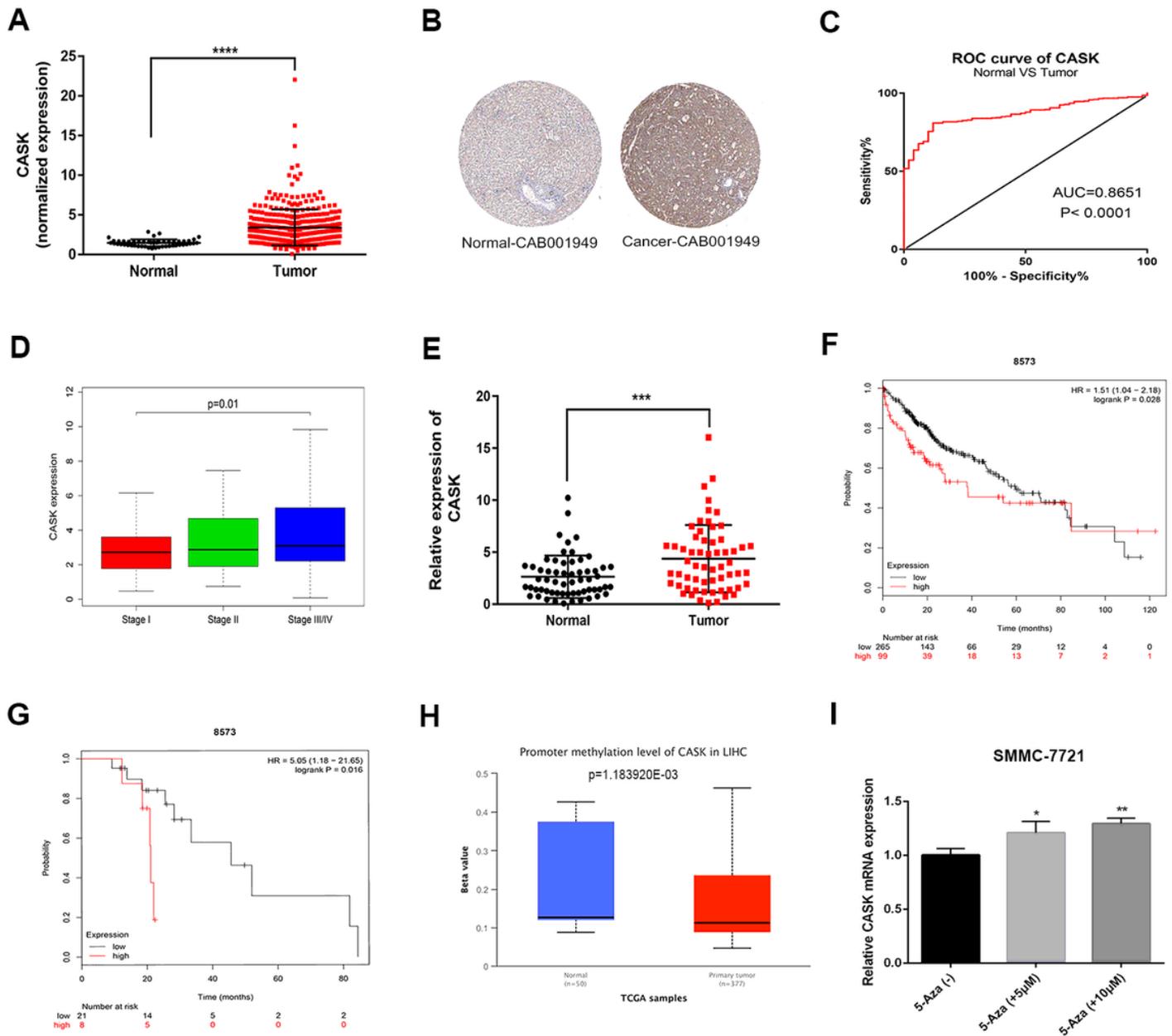
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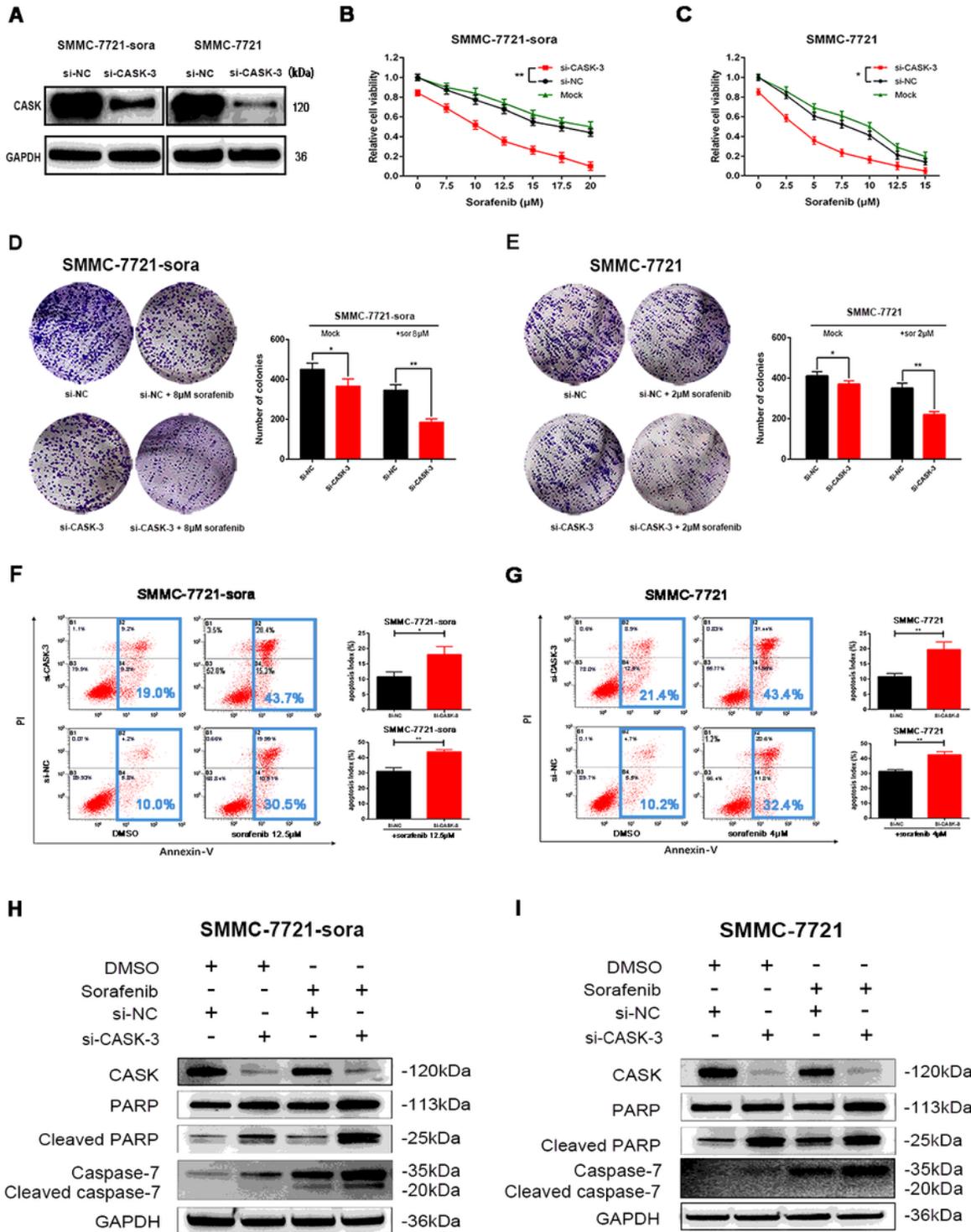
## Figures



**Figure 1**

CASK expression was elevated in HCC, and correlated with sorafenib resistance and poor prognosis. (a) Expression of CASK in HCC tissues were compared with normal tissues from TCGA database. (b) IHC of CASK expression in HCC tissue and normal tissue through using HPA database. (c) ROC curve analysis of CASK in HCC. (d) The correlation analysis between CASK levels and the stage in HCC cancer patients. (e) qRT-PCR analysis of CASK expression levels in 60 paired HCC tissues and corresponding normal tissues. (f) The Kaplan-Meier survival analysis of overall survival for HCC patients. (g) The Kaplan-Meier survival analysis of overall survival for HCC patients with sorafenib treated. (h) The promoter region methylation levels of CASK in HCC and normal tissues from UALCAN database (<http://ualcan.path.uab.edu/>). (i) qRT-

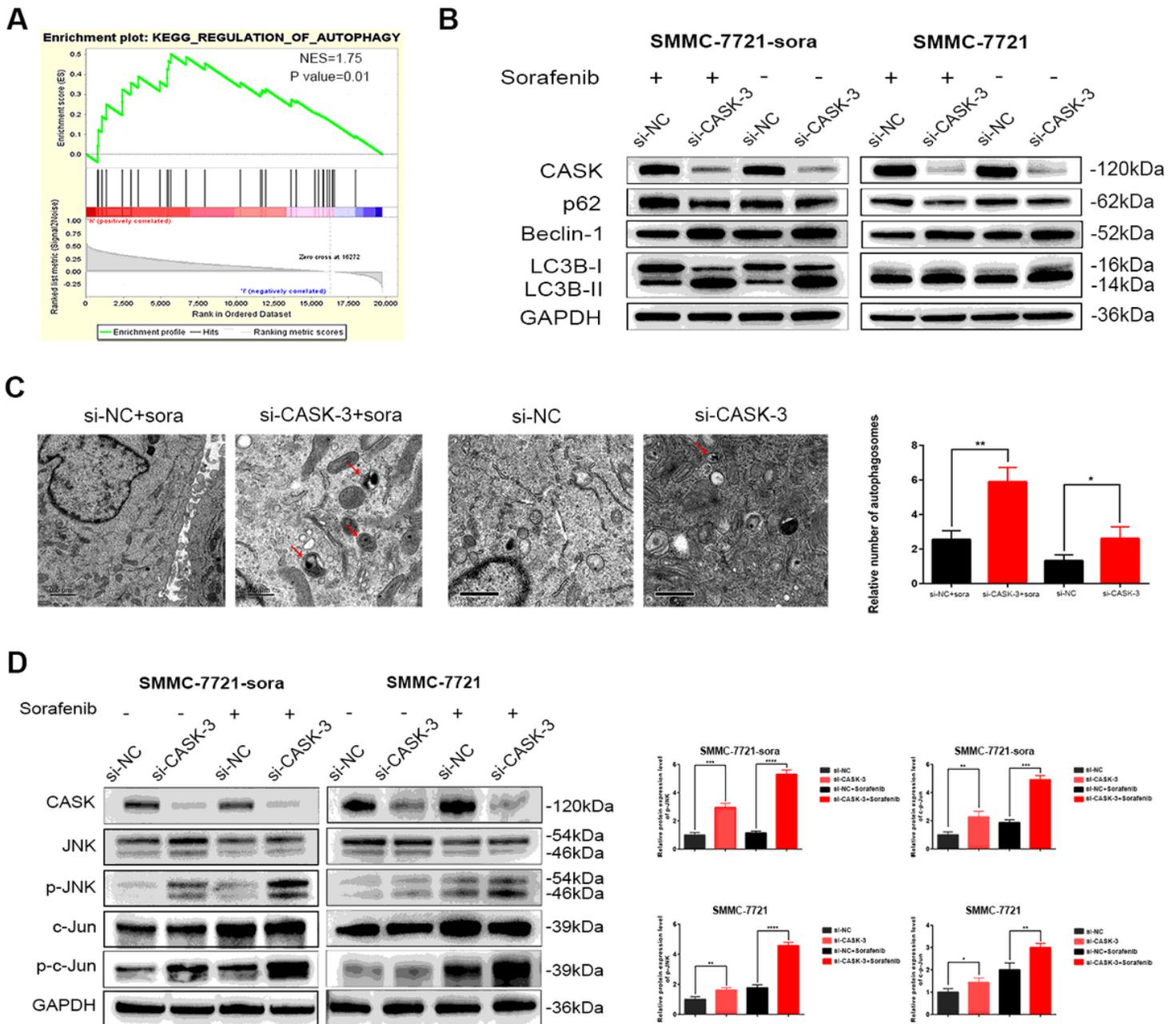
PCR analysis detecting the CASK levels in SMMC-7721 cells after treatment with 5 $\mu$ M 5-azacytidine (5-Aza) or 10 $\mu$ M 5-azacytidine (5-Aza) for 72h. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.



**Figure 2**

CASK downregulation inhibited HCC cell growth, promoted HCC cell apoptosis and attenuated sorafenib resistance. (a) The effect of si-CASK-3 in SMMC-7721-sora and SMMC-7721 cells were detected by performing western blotting. (b, c) CCK-8 assay analysis of the impact of CASK knockdown combine with

various concentrations of sorafenib on SMMC-7721-sora and SMMC-7721 cells growth. (d, e) Colony information assay showing the effects of CASK knockdown on SMMC-7721-sora and SMMC-7721 cells growth with or without sorafenib treated. (f, g) The apoptosis rate in CASK knockdown or control cells with or without sorafenib treated were measured by flow cytometry analysis. (h, i) Western blotting analysis of apoptosis-related protein levels in CASK knockdown or control cells, with or without sorafenib treated. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 3**

CASK depletion increased autophagy and activated JNK/c-Jun signaling pathway. (a) Enrichment plots from Gene Set Enrichment Analysis (GSEA). The GSEA results indicated that the regulation of autophagy

was differentially enriched in HCC with high CASK expression. (NES: normalized enrichment score) (b) Western blot analysis showing levels of autophagy-related proteins in CASK knockdown cells with the presence or absence of sorafenib (c) Transmission electron microscopy (TEM) shows the number of autophagosomes were evaluated in CASK knockdown cells compared with control cells. (d) Western blotting analysis of the impact of CASK knockdown on the activity of JNK and c-Jun in SMMC-7721-sora and SMMC-7721 cells treated with or without sorafenib for 72h. \*P<0.05, \*\*P<0.01.

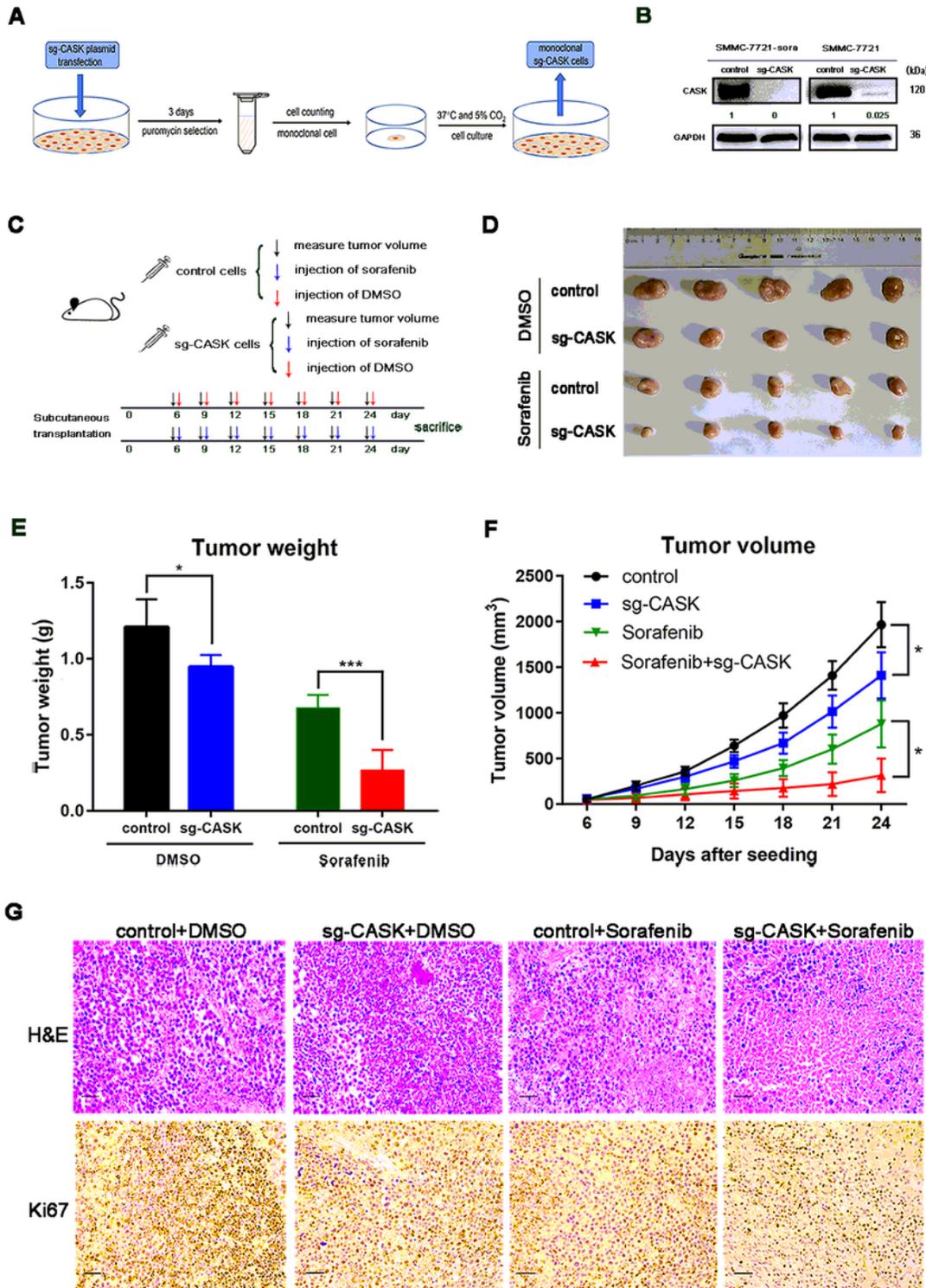
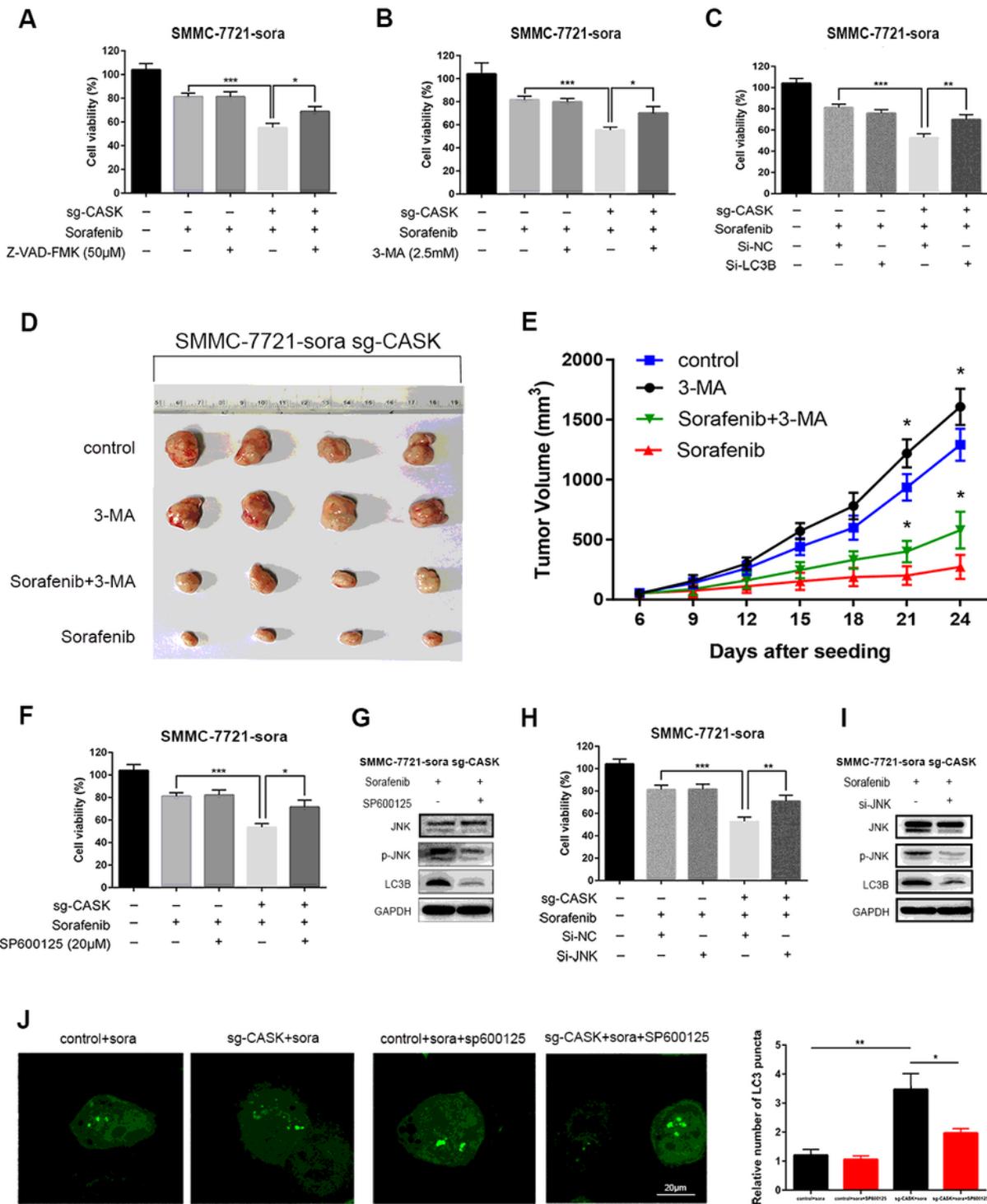


Figure 4

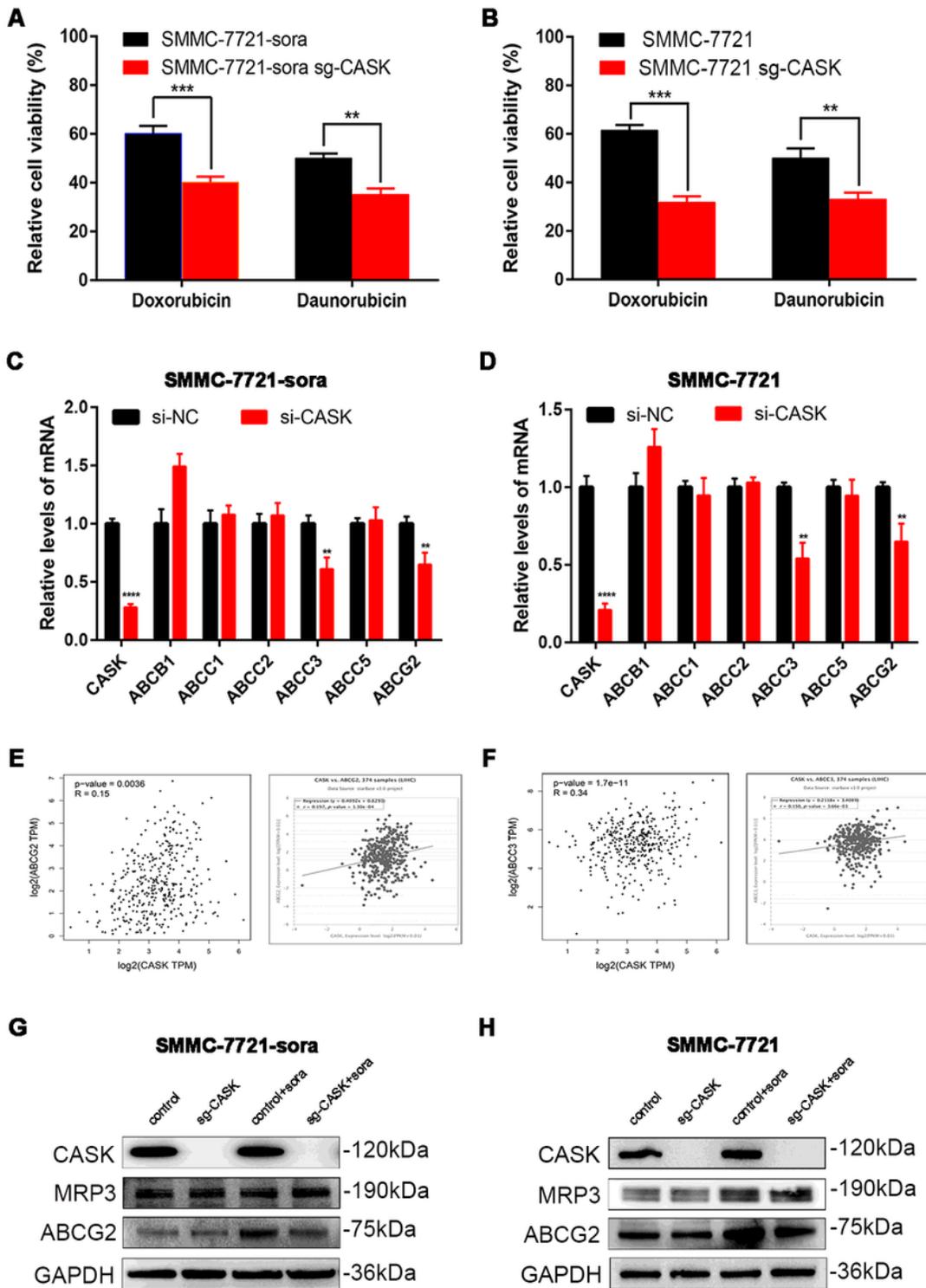
CASK knockout suppressed tumorigenesis and sorafenib resistance of HCC in vivo. (a) The flow chart for screening stable CASK knockout cell lines using CRISPR Cas9. (b) The western blot analysis indicates the protein expression of CASK in SMMC-7721-sora and SMMC-7721 cell lines stably-transfected by sgRNA against CASK using CRISPR Cas9. (c) Overall workflow of in vivo experiments. (d-f) The subcutaneous tumor models were built by SMMC-7721-sora with stable CASK knockout cells or control cells with following treatment of DMSO or sorafenib. The tumor weights and tumor volumes were measured and quantified. (g) Representative images of tumor samples with H&E and Ki67 staining. Scale bar: 100  $\mu$ M. \*P<0.05, \*\*\*P<0.001.



**Figure 5**

CASK depletion increased autophagic cell death through JNK/c-Jun signaling pathway. (a) Z-VAD-FMK partly attenuates CASK knockout-mediated sorafenib-induced HCC cell death by CCK8 assay. SMMC-7721-sora cell was pretreated with or without Z-VAD-FMK (50 µM) for 2h followed by exposure to 10µM sorafenib for 24h. (b) 3-MA partly attenuates CASK knockout-mediated sorafenib-induced SMMC-7721-sora cell death by CCK8 assay. Cells pretreated with or without 3-MA (2.5 mM) for 2h, and following

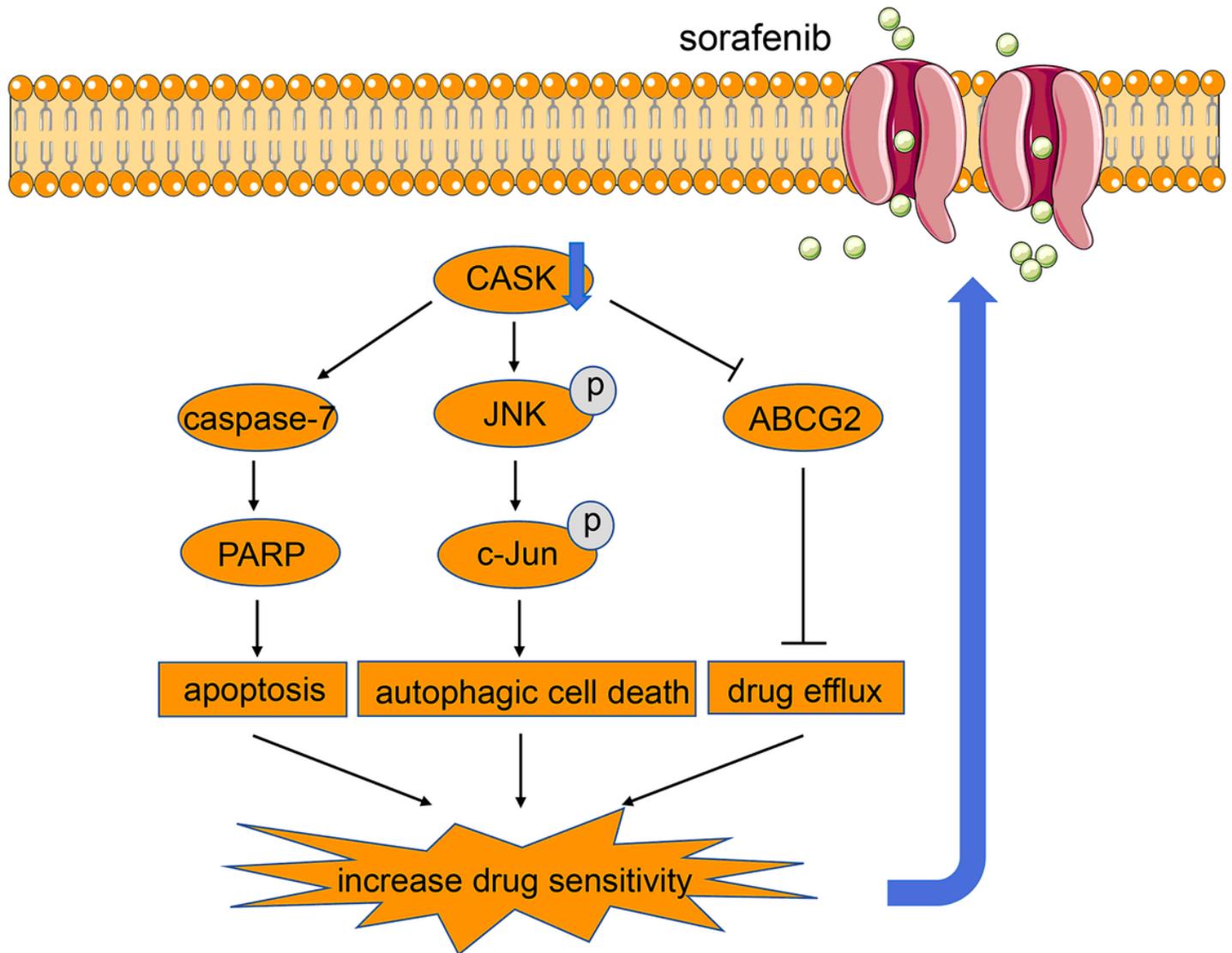
treated with 10 $\mu$ M sorafenib for another 24h. (c) si-LC3B partly reversed CASK knockout combine with sorafenib treatment-induced cell death by CCK8 assay. Cells pretreated with or without transiently transfected with si-LC3B for 12h, and incubated with 10  $\mu$ M sorafenib for another 72h. (d) Representative image of CASK knockout xenograft tumors. (e) Tumor volume in each group. (f) SP600125 partly decreased CASK knockout-mediated sorafenib-induced SMMC-7721-sora cell death by CCK8 assay. Cells pretreated with or without 20  $\mu$ M SP600125 for 2h, and following treated with 10  $\mu$ M sorafenib for another 24h. (g) Western blotting were performed to detect the expression of p-JNK and LC3B following the treatment of SP600125 in SMMC-7721-sora sg-CASK cell. (h) si-JNK partly decreased CASK knockout-mediated sorafenib-induced SMMC-7721-sora cell death by CCK8 assay. Cells pretreated with or without transiently transfected with si-NC/si-JNK for 12h and following treated with 10  $\mu$ M sorafenib for another 72h. (i) Western blotting were performed to detect the expression of p-JNK and LC3B following the treatment of si-JNK in SMMC-7721-sora sg-CASK cell. (j) CASK knockout or control cells transiently transfected with GFP-LC3 plasmid were pretreated with or without 20  $\mu$ M SP600125 for 2h. Then, cells were incubated with 12.5  $\mu$ M sorafenib for 36h. And the number of GFP-LC3 dots were visualized by confocal microscopy and quantified. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.



**Figure 6**

ABCG2 was involved in CASK-regulated multi-drug resistance of HCC cells. (a, b) The sensitivities of doxorubicin and daunorubicin in HCC cells were increased by CASK knockout. (c, d) mRNA expressions of ABC transporters were measured in SMMC-7721-sora and SMMC-7721 cells that transiently transfected with si-NC or si-CASK-3. (e, f) Correlation analysis between CASK and ABCC3, ABCG2 from GEPIA (<http://gepia.cancer-pku.cn/>) and starbase (<http://starbase.sysu.edu.cn>) database. (g, h) Western blotting

analysis of ABCG2 and MRP3 protein levels in CASK knockout or control cells, with or without sorafenib treated. \*\*P<0.01, \*\*\*\*P<0.0001.



**Figure 7**

Mechanistic model for CASK regulation of sorafenib-induced apoptosis and autophagy in HCC.

## Supplementary Files

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