YAP promotes sorafenib resistance in hepatocellular carcinoma by upregulating survivin

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Research

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Abstract

Background

Sorafenib is the standard first-line treatment for advanced hepatocellular carcinoma (HCC), but its use is hampered by the secondary drug resistance. Yes-associated protein (YAP) is the crucial downstream effector of the Hippo signaling pathway, which is crucial for liver tumorigenesis. However, the underlying mechanism regarding YAP and sorafenib resistance remains unclear.

Methods

Western blotting, flow cytometry, and CCK-8 assay were used to confirm the role of YAP in HCC sorafenib resistance. RT-PCR and western blotting were then performed to identify survivin as downstream of YAP, whereas rescue experiments were performed to confirm that YAP induces sorafenib resistance via survivin. Further, western blotting, flow cytometry, and an *in vivo* xenograft model were used to evaluate the function of verteporfin in combination with sorafenib in HCC.

Results

We found that sorafenib enhances YAP nuclear accumulation and activates YAP, which promotes sorafenib resistance by inhibiting apoptosis in HCC cells. Moreover, survivin acted as a downstream mediator of YAP to resist sorafenib-induced apoptosis. Pharmacological inhibition of YAP by verteporfin inhibited HCC cells proliferation and restored the sensitivity to sorafenib. Moreover, verteporfin in combination with sorafenib significantly suppressed HepG2 xenograft tumor growth.

Conclusions

Our study indicates that YAP promotes sorafenib resistance in HCC through upregulating survivin expression. Targeting YAP may be a potential therapeutic strategy to improve the antitumor effects of sorafenib in HCC.

Background

Liver cancer is one of the most frequent cause of cancer-related death worldwide, with a 5-year survival rate 18% [1]. Liver cancer incidence is rising faster than that for any other cancer in United States [2]. Hepatocellular cancer (HCC) comprises 90% of liver cancer cases [3]. China is one of the most high-risk regions for HCC [4]. HCC develops in patients with underlying chronic liver inflammation related to viral infection, alcohol, or metabolic syndrome. Despite efforts to elucidate the molecular mechanisms involved in HCC development and progression, the understanding of this disease is still limited and therapeutic effects are not satisfactory. Sorafenib, a multi-kinase inhibitor, remains the first-line treatment

for advanced HCC patients [5]. Although sorafenib has been shown to improve the overall survival of advanced HCC patients, the response rate is not satisfactory, and the development of sorafenib resistance often prevents its long-term efficacy [5–9]. Thus, a systematic understanding of the molecular mechanism associated with sorafenib resistance is critical to improve antitumor effect of sorafenib in HCC patients.

The transcriptional coactivator YAP is a crucial downstream effector of the Hippo signaling pathway, which plays an important role in organ size control, tissue homeostasis and cancer [10–12]. YAP can be regulated through phosphorylation by the core MTS1/2-LATS1/2 kinase cascade [13]. Mounting evidence suggests that aberrant YAP expression or activity are involved in cancer initiation and progression [12, 14]. It has been reported that 5–10% of human HCC have YAP amplification on the chromosome 11q22 amplicon [15] and approximately 60% of human liver cancers associated with increased YAP activity [16, 17]. Studies have also shown that YAP is critical for liver tumorigenesis [18–21]. In addition, YAP also promote resistance to targeted therapy. Lin et al. reported that YAP promotes resistance to RAF and MEK inhibitors in several cancer cell lines harboring BRAF, KRAS, or NRAS activating mutations [22]. However, the role of YAP in sorafenib resistance remains unclear. Therefore, understanding of the molecular mechanism of YAP involved in sorafenib resistance may provide us with new insight to improve HCC response to sorafenib.

In this study, we explored the role of YAP in sorafenib resistance of HCC. Our study indicates that YAP contributes sorafenib resistance through upregulating survivin expression. Targeting YAP may be a potential therapeutic strategy to improve the antitumor effects of sorafenib in HCC.

Material And Methods

Cell culture and reagents

Huh-7, HepG2 and LO2 cells were obtained from the American Type Culture Collection. These cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 U/mL streptomycin. Cultures were maintained at 37℃ in a humidified at mosphere with 5% CO2. Sorafenib and Verteporfin were purchased from Selleck Chemicals. Antibody to PARP, YAP, p-YAP(S127), survivin, Bcl-xl and Histone H3 were purchased from Cell Signaling Technology, Inc.; antibody to GAPDH was from Santa Cruz Biotechnology, Inc.; antibody to Flag was from Sigma.

Cell viability assay

Cells were seeded in 96-well plates (4,000 cells/well) and incubated overnight for attachment, and were then treated with indicated agents in 10% FBS-supplemented medium for 72 hours. The medium was replaced with CCK-8 at 37℃ for 2 hours and absorbance at 450 nm was measured.

Immunofluorescence assay

For immunofluorescence analysis, cells were plated in chamber slides then fixed in methanol for 10 min at room temperature, permeabilized with 5% bovine serum albumin in PBST. Cells were then exposed to primary antibodies (anti-YAP 1:200) diluted in PBST containing 5% bovine serum albumin overnight at 4°C. After washing three times with PBS for 10 min, secondary antibody (Alexa Fluor 488- goat anti-rabbit 1:200) diluted in PBST was added and incubated for 1 h at room temperature. Cells were then washed in PBS and mounted using 4,6-diamidino-2-phenylindole (DAPI) to counterstain DNA. Images were collected using a confocal microscope (Olympus FV-1000).

Colony formation assay

For Colony formation, the cells were seeded into 6-well plates (500 cells per well), and then treated with sorafenib and verteporfin, alone or in combination. The medium was replaced with fresh medium containing the reagent every three days. After 10 days treatment, the medium was removed and cell colonies were fixed with 4% paraformaldehyde for 20 minutes and stained with crystal violet (0.1% in 20% methanol) for 30 minutes. Then they were washed slowly with running water and air dried naturally. Pictures were taken using a digital camera to record the result, and the number of cell clones with more than 50 cells was counted under the microscope.

Plasmids and transfection

Plasmids encoding the human YAP and survivin were cloned into pcDNA3.1 vector with the Flag-tag. For transient transfection, plasmids were pretransfected with lipofetamine 2000 (Invitrogene) for 24 hours and then processed with the indicated treatment as described.

RNA interference

Target siRNA was produced by GenePharma (Suzhou, China) and transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogene) according to the manufacturer’s protocol. A non-targeting siRNA was used as a negative control. Target sequence as follows: survivin (5'-AAGGAGAUCAACAUUUUCA-3').

For stable YAP knockdown, the following Addgene plasmids were used, pLKO1-shYAP#1(27368) and pLKO1-shYAP#2 (27369).

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIZOL Reagent (Invitrogen), and then reverse transcribed to cDNA with M-MLVReverse Transcriptase (Promega). Real-time PCR was carried out by FastStart Universal SYBR Green Master (Roche) and cDNA amplification was detected by the StepOne RT-PCR System (Applied Biosystems). The primers used were as follows:

CTGF-forward: 5'AGGAGTGGGTGTGTGACGA3'

CTGF-reverse: 5'CCAGGCAGTTGGCTCTAATC3';

CYR61-forward: 5'CCTTGTGGACAGCCAGTGTA3'

CYR61-reverse: 5'ACTTGGGCCGGTATTTCTTC3';

Survivin-forward: 5'GAGGCTGGCTTCATCCACTG3'

Survivin-reverse: 5'ATGCTCCTCTATCGGGTTGTC3';

GAPDH-forward: 5'CTCCTGCACCACCAACTGCT3'

GAPDH-reverse: 5'GGGCCATCCACAGTCTTCTG3'.

Flow cytometric analysis of apoptosis

Apoptotic rate was detected by flow cytometry with the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences). Briefly, cells were collected after different treatment and the assay were performed according to the manufacturer's instruction. Samples were analyzed immediately using a Cytomics FC500 flow cytometer (Beckman Coulter).

Xenograft tumor growth

For the subcutaneous xenograft tumor model, each nude mice (nu/nu, 5-week-old females) were injected subcutaneously in the dorsal flank with 5×10^6 HepG2 cells suspended in 0.1 mL of serum-free medium. When tumors reached 100 to 200 mm^3, mice were randomly divided into four groups, and received vehicle, sorafenib (50 mg/kg) orally once daily, verteporfin (100 mg/kg) intraperitoneally every other day or the combination of sorafenib and verteporfin respectively. Tumor volume was measured every 4 days using calipers and their volumes calculated using the following formula (tumor volume $=\pi/6\ (L\times W^2)$). Mice were sacrificed on day 32, and the tumors were dissected and analyzed.

Immunohistochemistry (IHC)

Xenograft tumors were fixed in 4% PFA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Immunohistochemical staining of paraffin-embedded tumor tissues was performed using survivin (CST, 1:100 dilution) and Ki-67 (Abcam, 1:100 dilution) primary antibodies and the ABC Elite immunoperoxidase kit according to the manufacturer’s instructions.

Statistical analysis

The data were analyzed using SPSS 16.0 software. The Student’s t test was calculated to compare the mean of different groups and $p < 0.05$ were considered significant.

Results

Sorafenib induces apoptosis and activates YAP in HCC cells

Sorafenib has the potential of inhibiting tumor growth, progression, metastasis, and angiogenesis [23]. We found that sorafenib induced apoptosis in Huh-7 and HepG2 cells and cleaved PARP levels were strongly increased in a dose- and time- dependent manner (Fig. 1A-D). Previous work indicates that YAP plays an important role in tumorigenesis by regulating cell proliferation and apoptosis [10, 24, 25]. Thus, we evaluated the YAP expression levels after sorafenib treatment in HCC cells. Western blotting showed no detectable difference in total YAP protein levels, but a substantial decrease in YAP Ser127 phosphorylation after sorafenib treatment (Fig. 1A-D). YAP phosphorylation on Ser127 mediated by Hippo pathway mainly leads to its cytoplasm sequestration and degradation [26]. We also assessed the localization of YAP by immunofluorescence assay and found that sorafenib significantly increased the nuclear accumulation of YAP (Fig. 1E-F). Furthermore, we separated proteins from the cytoplasm and nucleus, and western blotting also confirmed the more YAP nuclear accumulation after sorafenib treatment (Fig. 1G-H). To further confirm the activation of YAP in the sorafenib treated cells, we measured YAP target gene expression. Sorafenib significantly increased the expression of CTGF and CYR61, TEAD target genes (Fig. 1I-J). Altogether, these data indicated that sorafenib promotes YAP nuclear accumulation and activates YAP in HCC cells.

YAP promotes sorafenib resistance by inhibiting apoptosis

Previous work indicates that YAP can regulate apoptosis [27, 28]. To investigate the biological significance of YAP in sorafenib-induced apoptosis, we used independent shRNAs to knockdown YAP in Huh-7 and HepG2 cells. We found that YAP knockdown promotes sorafenib-induced apoptosis, as measured by both the induction of PARP cleavage and caspase activity in HCC cells (Fig. 2A-C). In contrast, YAP overexpression inhibited sorafenib-induced apoptosis in LO2 cells (Fig. 2D-E). LO2 is a normal hepatocyte cell line, expressing very low level of endogenous YAP. We also examined sorafenib-induced apoptosis by performing flow cytometry using Annexin V-FITC/PI double staining. Apoptosis cell rates were assessed 48 hours after sorafenib treatment. YAP knockdown increased about three-fold of the apoptotic cells in Huh-7, while YAP overexpression reduced the apoptotic cells by 60% in LO2 (Fig. 2H-I). Since YAP could suppress sorafenib-induced apoptosis, we hypothesized that YAP may promote sorafenib resistance in HCC cells. Cell viability was measured by CCK-8 assay. YAP knockdown enhanced the cytotoxicity of sorafenib in Huh-7 cells (Fig. 2F). On the contrary, YAP overexpression reduced the cytotoxicity of sorafenib in LO2 cells (Fig. 2G). Collectively, these data demonstrate that YAP plays a crucial role in mediating sorafenib resistance in HCC cells.

Survivin acts as a downstream mediator of YAP in sorafenib-induced apoptosis

We next investigated the mechanism through which YAP regulates sorafenib resistance. It has been reported that YAP can transcriptionally upregulate the expression of specific antiapoptotic components, including BCL-xL [22, 29] and survivin [30, 31] in some cell types. We reasoned that YAP might enhance the expression of antiapoptotic factors to promote tumor cells survival and sorafenib resistance in HCC cells. Indeed, YAP knockdown resulted in decreased expression of survivin, both the protein and mRNA level, specifically with sorafenib treatment in Huh-7 and HepG2 cells (Fig. 3A-D). On the contrary, YAP

overexpression increased survivin transcription and expression (Fig. 3E-F). However, the level of BCL-xL was not dramatically affected, indicating a potential link between YAP and survivin in the sorafenib treated HCC cells.

To further prove the functional link between YAP and survivin, cells were treated with small interfering RNA (siRNA) to knockdown survivin expression. Our data clearly showed that survivin silencing significantly increase sorafenib-induced PARP cleavage in Huh-7 and HepG2 cells (Fig. 3G). Moreover, survivin knockdown rescued the inhibitory function of YAP overexpression upon apoptosis in LO2 cells (Fig. 3H), while survivin overexpression reduced sorafenib-induced apoptosis promoted by YAP knockdown in HepG2 cells (Fig. 3I). Together, these data support the notion that survivin serves as a downstream mediator of YAP in sorafenib-induced apoptosis.

Verteporfin increases the sensitivity of HCC cells to sorafenib

Verteporfin, a photosensitizer clinically used in photodynamic therapy, abrogates the interaction between YAP and TEAD, thereby inhibiting YAP transcriptional activity [32]. Therefore, we hypothesized that verteporfin may reverse sorafenib resistance by inhibiting YAP. HCC cells were treated with sorafenib or synergized with verteporfin. We found that, compared with sorafenib alone, the combination of verteporfin and sorafenib significantly decreases survivin expression and enhances PARP cleavage (Fig. 4A-B). Cell viability analysis showed that verteporfin significantly increased the antitumor activity of sorafenib in Huh-7 and HepG2 cells (Fig. 4C-D). Furthermore, the combination of verteporfin and sorafenib resulted in a marked reduction in colony formation of HCC cells compared with verteporfin or sorafenib only (Fig. 4E-F). Verteporfin also significantly enhanced sorafenib-induced apoptosis in Huh-7 and HepG2 cells (Fig. 4G). These findings indicated that verteporfin could enhance sorafenib-induced apoptosis and increase sensitivity of HCC cells to sorafenib.

Verteporfin enhances the antitumor activity of sorafenib in vivo

To confirm whether the synergistic effect of sorafenib and verteporfin has potentially relevant clinical implications, we evaluated the effect of verteporfin on the antitumor activity of sorafenib *in vivo.* BALB/c (nu/nu) mice were injected subcutaneously with HepG2 cells and divided randomly into 4 groups. Tumor-bearing mice were treated with vehicle, sorafenib (50 mg/kg) orally once daily, verteporfin (100 mg/kg) intraperitoneally every other day or the combination of sorafenib and verteporfin. All animals tolerated the treatments well without observable signs of toxicity and had stable body weights throughout the course of study. As shown in Fig. 5A and 5B , the combination of sorafenib and verteporfin significantly delayed tumor growth compared with sorafenib or verteporfin alone. Accordingly, immunohistochemical staining revealed that survivin and Ki67 were dramatically diminished by the combination treatment of sorafenib and verteporfin (Fig. 5C). Thus, sorafenib in combination with verteporfin exhibited better antitumor activity *in vivo* and these effects were, at least in part, due to the inhibition of YAP and survivin.

Discussion

Liver cancer is one of the leading causes of cancer-related death worldwide and currently has the fastest rising incidence of all cancers. Although sorafenib represents the standard first-line treatment for advanced HCC, sorafenib resistance is a unique concern due to the shortage of alternative systemic treatments for HCC [33]. This study was the first to identify the role of YAP in HCC with regard to sorafenib resistance. We demonstrated that activated YAP in HCC cells correlated with therapeutic effect of sorafenib. Survivin was found to be a critical mediator of YAP in sorafenib resistance. The combination of the YAP inhibitor verteporfin can significantly improve the antitumor activity of sorafenib both *in vitro* and *in vivo*. These findings suggest that inhibiting YAP might be an ideal strategy to improve the efficacy of sorafenib in HCC.

Research over the past decade has demonstrated the critical role of the Hippo/YAP pathway in organ development and cancer. There is no doubt that YAP is emerging as an attractive therapeutic target for cancer. Previous studies have shown that YAP contributes to the development of cancer resistance [34, 35]. However, the direct relationship between YAP and sorafenib resistance in HCC is currently poorly understood. Here, we demonstrated that YAP promotes sorafenib resistance and YAP inhibition enhances the antitumor activity of sorafenib. Notably, we identified survivin as a crucial mediator of YAP in sorafenib resistance of HCC. Survivin is a member of the inhibitor of apoptosis protein family widely overexpressed in human cancers and well known for driving evasion from apoptosis. Thus, our findings not only confirm the regulatory relationship between YAP and survivin, but also provide evidence that survivin serves as an executor of YAP in promoting sorafenib resistance.

Targeting YAP has become an exciting yet challenging goal for cancer therapy. Verteporfin is a small molecule that inhibits YAP-TEAD interaction, and several studies have used it as an effective YAP inhibitor to suppress YAP-induced tumorigenesis [36–38]. Recently drugs such as metformin and statins, have been shown to effectively target upstream pathways and indirectly inhibit YAP activity [39, 40], indicating inhibition of YAP may become clinically feasible.

Conclusions

Collectively, our study demonstrated that YAP promotes sorafenib resistance in HCC through downstream regulation of survivin. It suggests that the combination of YAP inhibitors with sorafenib may be a promising therapeutic strategy for advanced HCC.

Abbreviations

HCC: Hepatocellular cancer; IHC: Immunohistochemistry; RT-PCR: real-time polymerase chain reaction; siRNA: small interfering RNA; YAP: Yes-associated protein

Declarations

Funding

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Availability of data and materials

The material supporting the conclusion of this review has been included within the article.

Authors' contributions

TS directed the study and prepared the manuscript; WM performed most of the experiments; LJ and JW participated in some experiments; TS and WM analyzed the data and competed the figures; LM revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were in accordance with the ethical standards of the ethics committee of the First Affiliated Hospital of Zhengzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

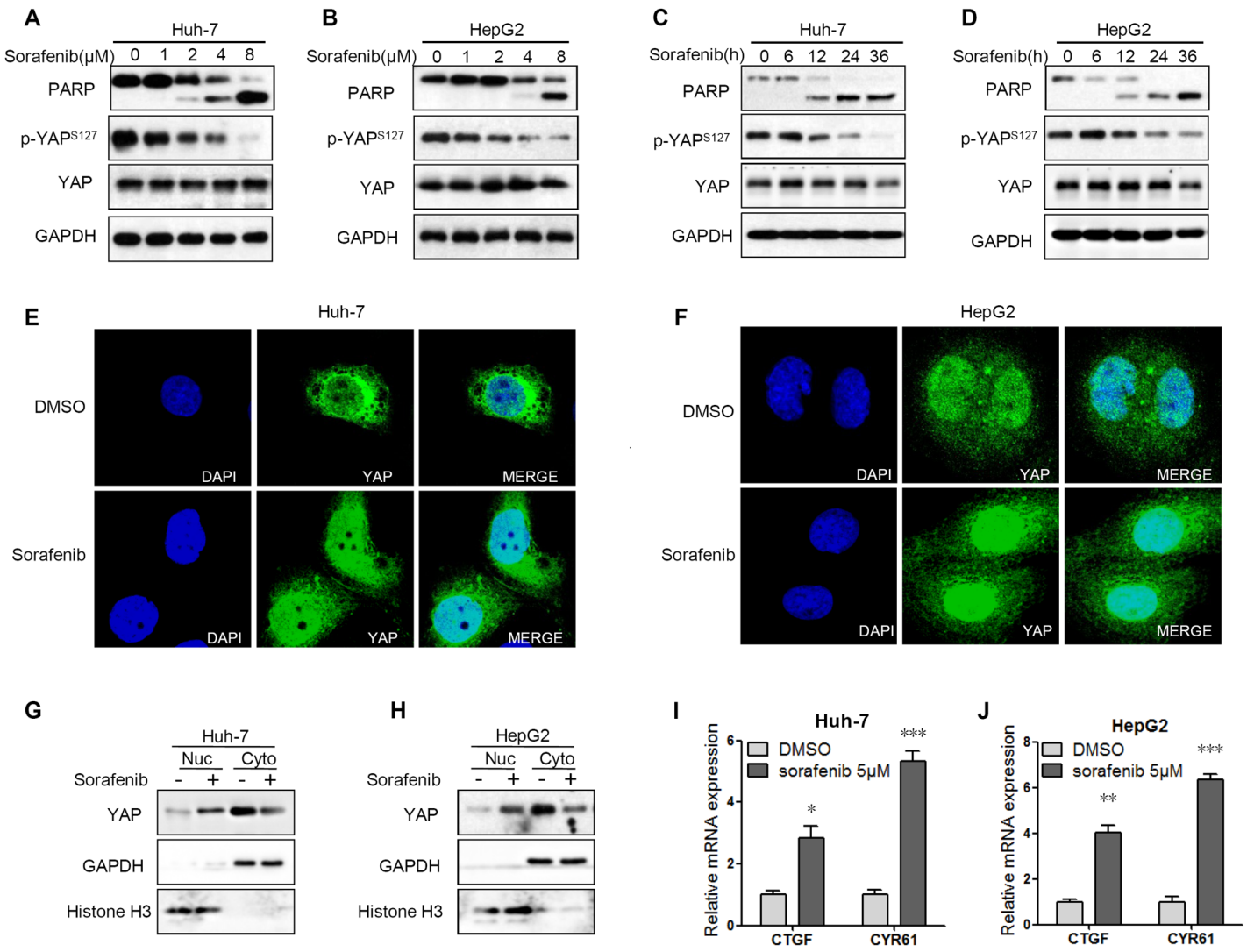

Figure 2

Sorafenib promotes YAP nuclear accumulation and activates YAP in HCC cells. (A, B) Huh-7 and HepG2 cells were exposed to the indicated doses of sorafenib for 24 h. Cells were collected for western blotting. (C, D) Huh-7 and HepG2 cells were treated with 5 μM sorafenib for indicated times. Cells were collected for western blotting. (E, F) The localization of YAP were detected using immunofluorescence assay. (G, H) Proteins from the cytoplasm and nucleus were separated, and the localization of YAP were detected by western blotting. (I, J) The mRNA expressions of CTGF and CYR61 were analyzed by qRT-PCR. Results were presented as mean ± sem (n=3) for each treatment. *p < 0.05, **p < 0.01, ***p < 0.001.

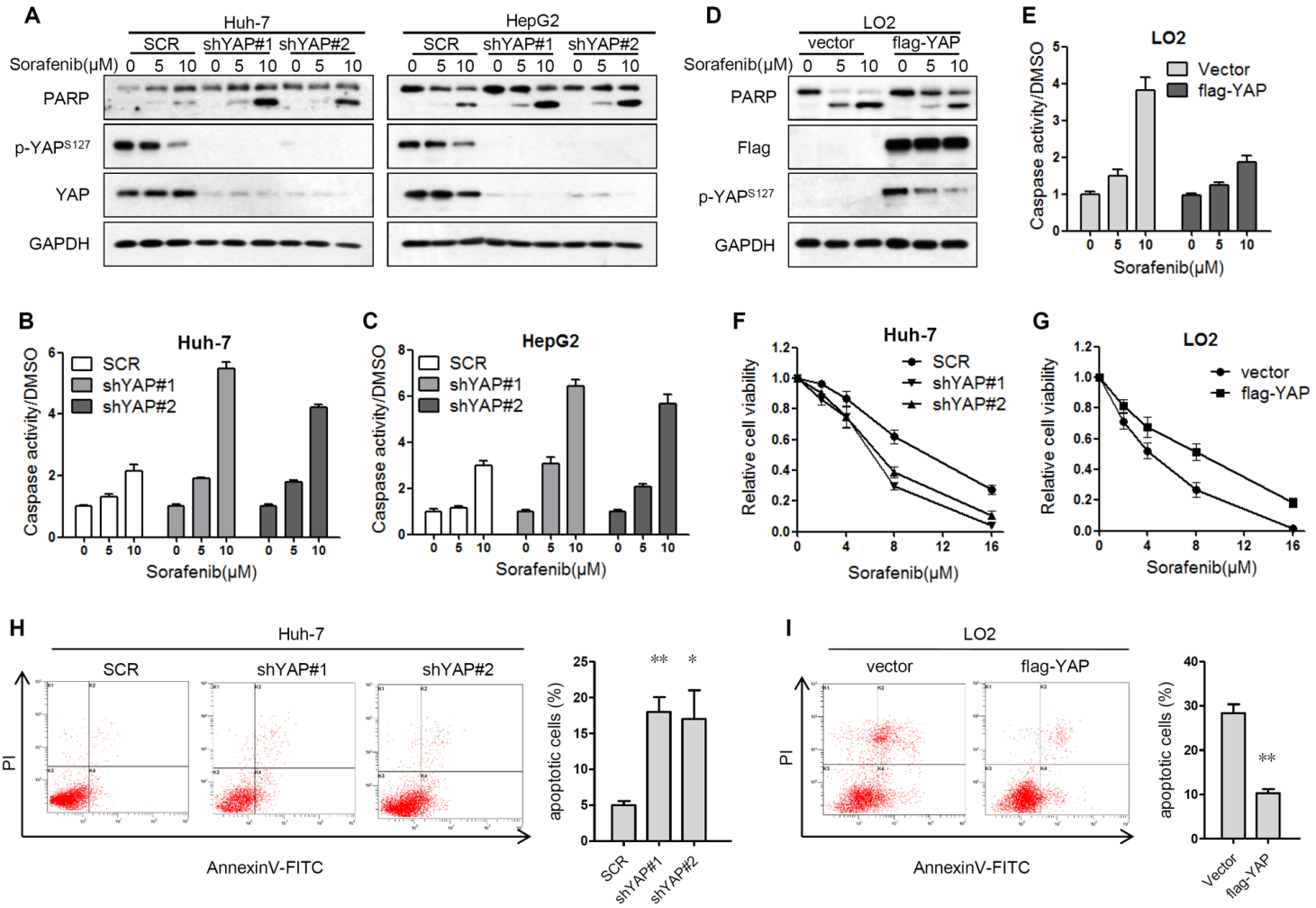

Figure 4

YAP induced sorafenib resistance in HCC cells. (A-C) Huh-7 and HepG2 cells transfected with shYAP were treated with sorafenib for 24 h. Proteins were analyzed with western blotting and the activity of caspase-3 and caspase-7 were detected. (D, E) LO2 cells transfected with flag-YAP plasmids were treated with sorafenib for 24 h. Proteins were analyzed with western blotting and the activity of caspase-3 and caspase-7 were detected. (F, G) CCK-8 assays were performed to detect the growth inhibition of sorafenib on Huh-7 with YAP knockdown and LO2 with YAP overexpression. (H, I) Huh-7 cells with YAP knockdown and LO2 cells with YAP overexpression were treated with sorafenib and cells were analyzed by flow cytometry (Left panels). Columns, representing the total percentage of K2 and K4, were the average of three independent experiments (Right panels). Results were presented as mean ± sem (n=3). *p < 0.05, **p < 0.01, ***p < 0.001.

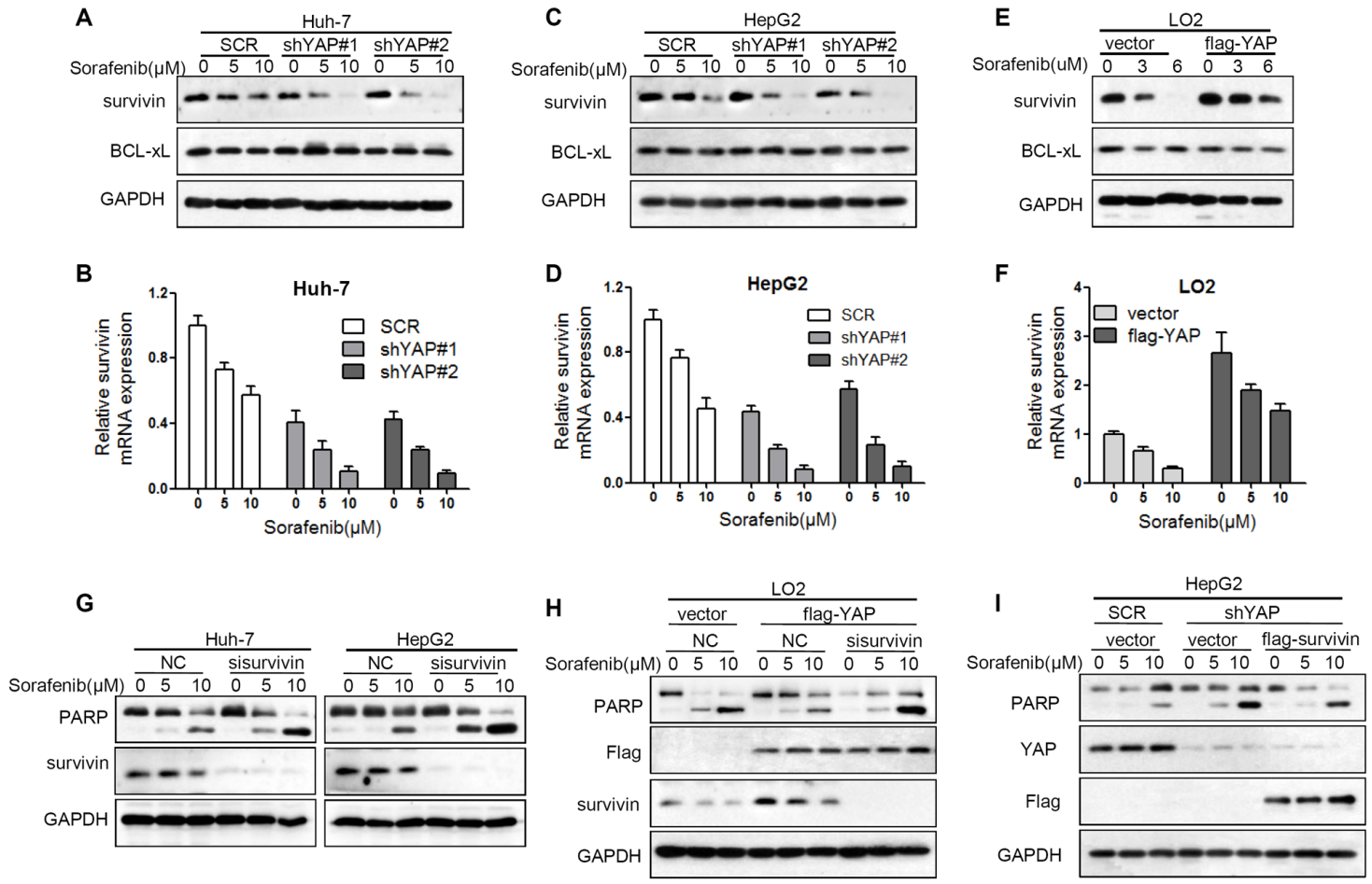

Figure 6

YAP promoted the expression of survivin to inhibit sorafenib-induced apoptosis. (A-D) Huh-7 and HepG2 cells transfected with shYAP were treated with sorafenib for 24 h. Protein and mRNA expressions were analyzed by western blotting and qRT-PCR. (E, F) LO2 cells transfected with flag-YAP were treated with sorafenib for 24 h. Protein and mRNA expressions were analyzed by western blotting and qRT-PCR. (G) Huh-7 and HepG2 cells transfected with sisurvivin were treated with sorafenib for 24 h and cells were analyzed using western blotting assay. (H) LO2 cells transfected with different combinations of flag-YAP and sisurvivin were treated with sorafenib and cells were analyzed using western blotting. (I) HepG2 cells transfected with different combinations of shYAP and flag-survivin were treated with sorafenib and cells were analyzed using western blotting.

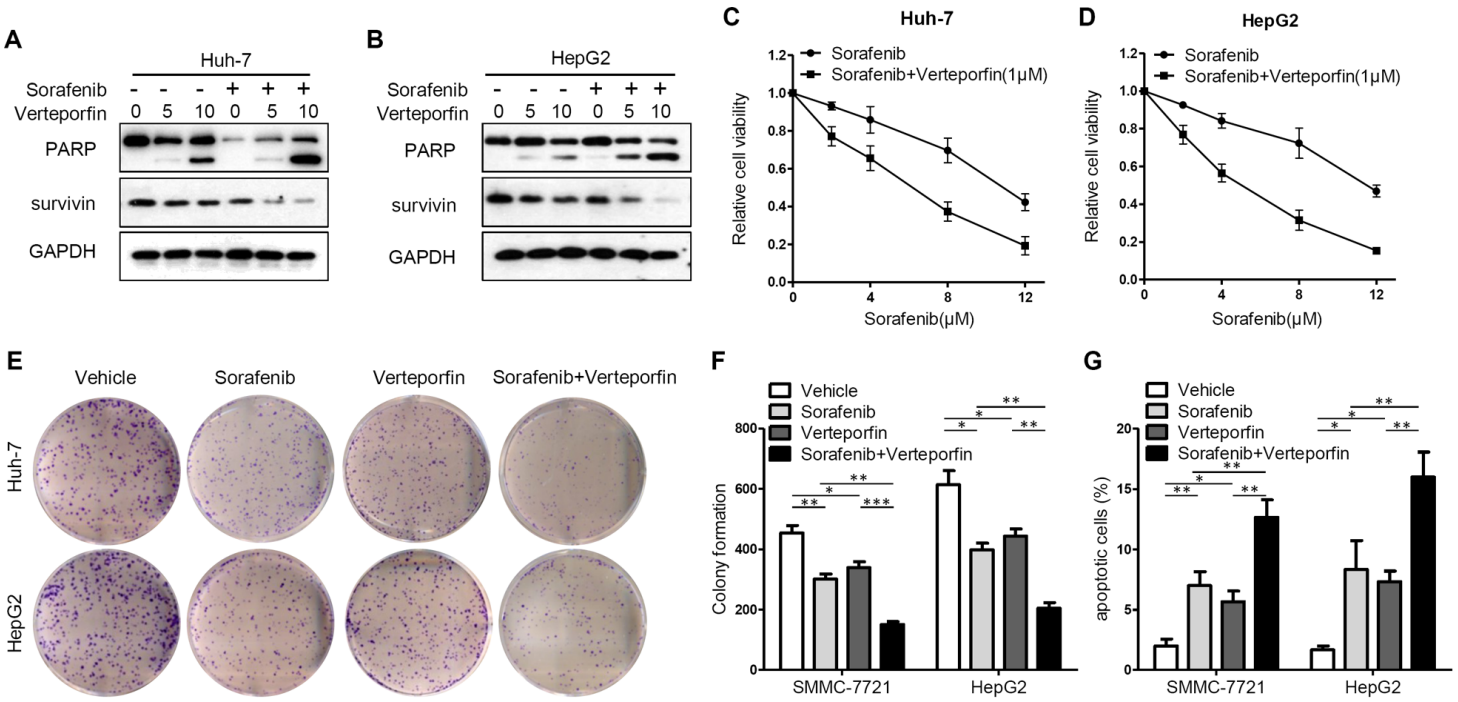

Figure 8

YAP inhibitor verteporfin promoted sorafenib cytotoxicity in HCC cells. (A, B) Huh-7 and HepG2 cells were treated with sorafenib in combination with or without verteporfin for 24 hours. Cells were analyzed using western blotting. (C, D) Huh-7 and HepG2 cells were treated with different combinations of sorafenib and verteporfin for 72 h. Cell viability was measured by CCK-8. (E, F) HepG2 and Huh-7 cells were treated with different combinations of sorafenib and verteporfin. Cells viability was analyzed by colony formation assay. Columns were the average of three independent experiments. (G) HepG2 cells were treated with different combinations of sorafenib and verteporfin for 48 h. Cells were analyzed by flow cytometry. Columns were the average of three independent experiments. Results were presented as mean ± sem (n=3). *p < 0.05, **p < 0.01, ***p < 0.001.

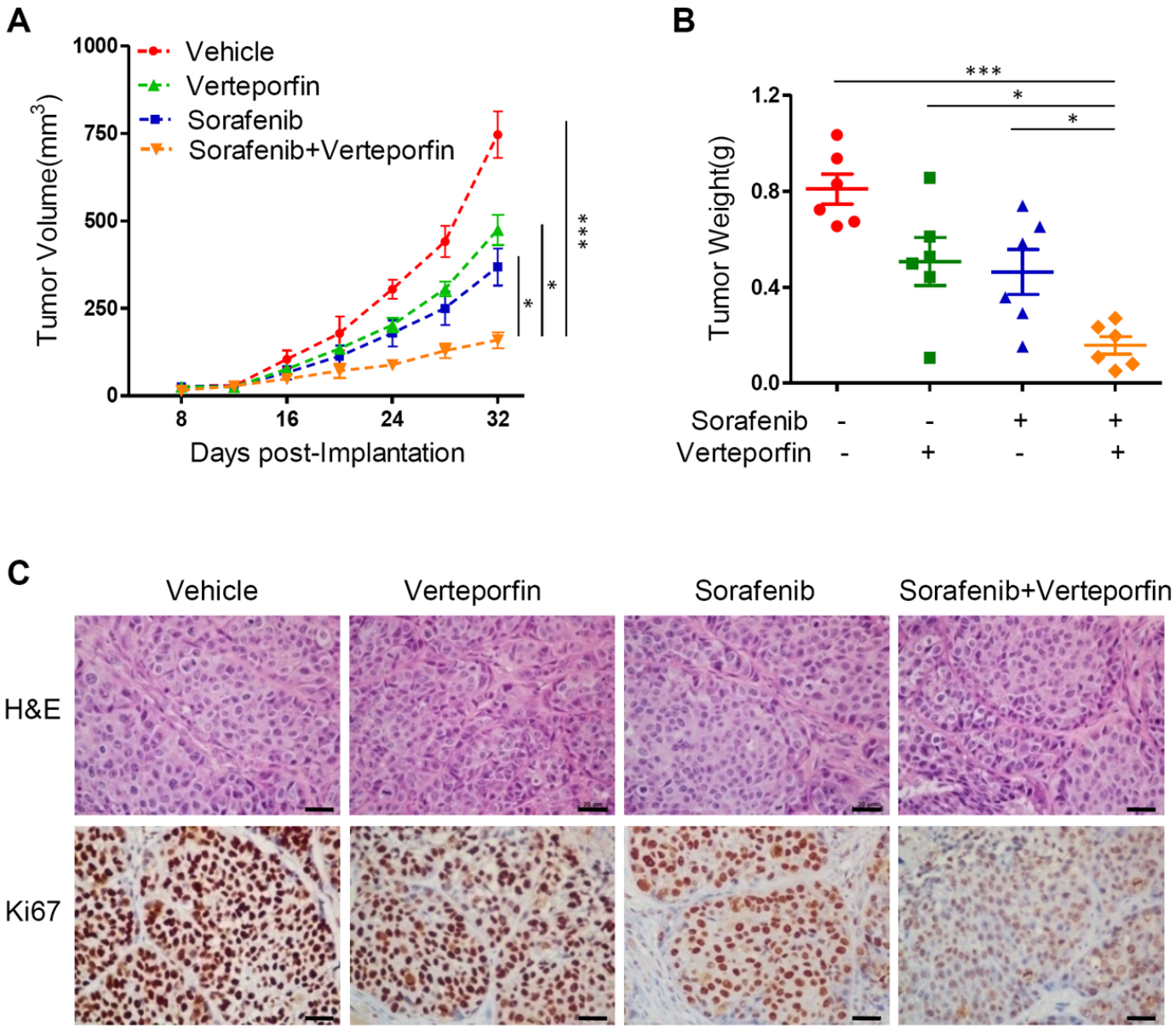

Figure 10

Verteporfin enhances the anti-cancer effect of sorafenib in HepG2 xenograft models. (A) HepG2 cells were subcutaneously implanted in nude mice, and mice bearing tumors xenografts were treated as described in Materials and methods. Curves of tumor growth in each group were measured. (B) Tumor weights were measured after collection. (C) HE-stain and immunohistochemical stain for Ki-67 using xenograft tumor samples from each group. Scale bar = 20 μm. Results were presented as mean ± sem (n = 6) for each group. *p < 0.05, **p < 0.01, ***p < 0.001.

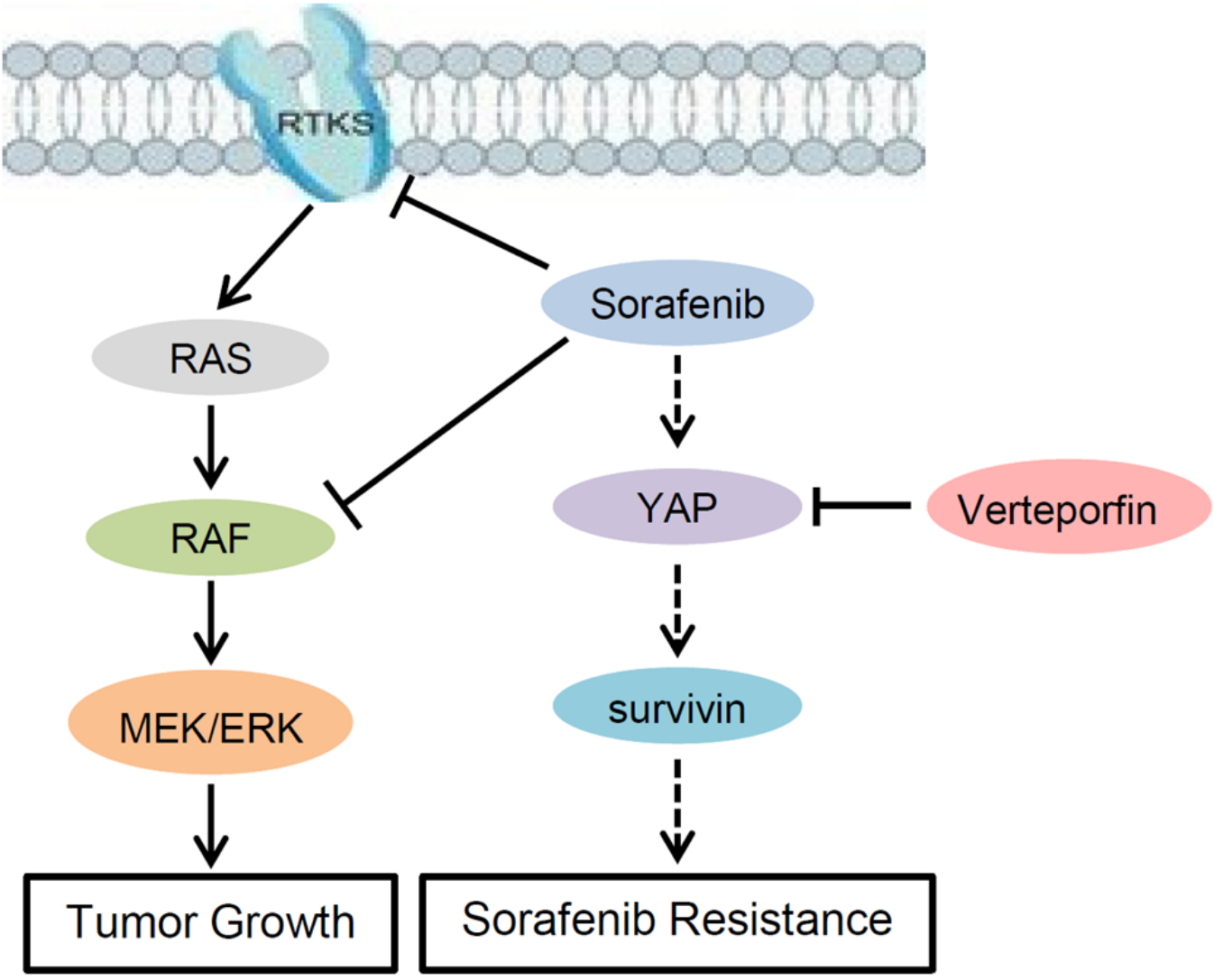

Figure 12

The diagram of YAP-mediated sorafenib resistance in HCC. Sorafenib suppresses tumor growth by inhibiting the RAS/RAF/MEK/ERK pathway. Meanwhile, sorafenib activates YAP, which contributes to sorafenib resistance by increasing the expression of survivin.