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Original Article

Combination of sorafenib and Valproic acid synergistically induces cell apoptosis and inhibits hepatocellular carcinoma growth via down-regulating Notch3 and pAkt

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Abstract: Sorafenib is currently the only approved first-line targeted drug against advanced hepatocellular carcinoma (HCC). However, unsatisfactory efficacy and resistance of sorafenib raises the urgent need to develop more effective therapeutic strategies for HCC. Here, we evaluated the effects of combination of histone deacetylase inhibitor Valproic acid (VPA) and sorafenib in HCC both in vitro and in vivo. Co-treatment of sorafenib and VPA synergistically inhibited HCC cell viability, induced cell apoptosis, along with the up-regulation of p21, Bax, cleaved caspase9, cleaved caspase3, cleaved PARP and down-regulation of Bcl-xL, suggesting this combination activated intrinsic apoptotic pathway. Our further experiment results showed that sorafenib plus VPA decreased tumor burden more effectively than sorafenib or VPA mono-therapy in nude mice subcutaneous xenograft model. Histological and serological analysis demonstrated well tolerance of this combination in vivo. On a molecular level, our results presented a possible crosstalk between Notch3 and Akt signaling. Sorafenib increased the expression of Notch3 in a dosage dependent manner, along with the phosphorylation of Akt in HCC cells. In comparison, this induction of Notch3 and pAkt could be decreased by VPA, implying that Notch3 and pAkt are of significance in the treatment of HCC, which may account for the synergism of sorafenib and VPA. In conclusion, the combination of sorafenib and VPA offers a potential targeting therapeutic regimen for HCC in the future.

Keywords: Hepatocellular carcinoma, sorafenib, valproic acid, synergistic, Notch3, Akt

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies with its mortality ranked second in male and sixth in female respectively in cancer-related deaths worldwide [1, 2]. The incidence of HCC is accelerating in many parts of the world including USA and Europe [3, 4], making it a major public health concern. Presently, sorafenib is the standard systemic treatment for patients with advanced hepatocellular carcinoma, which acts via inhibiting multiple kinases including Raf, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and c-Kit [5, 6]. However, the average lifetime extension of sorafenib is nearly 3 months and acquired resistance usually arises less than 6 months [5, 7, 8]. As a result, identification of agents that can synergistically potentiate anti-tumor effects of sorafenib without significant systemic toxicity is crucial to the development of effective therapies for HCC.

Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been a clinically established anti-epileptic drug for decades. It should be mentioned that VPA also has wide range of anti-tumor effects including glioma, prostate cancer, bladder cancer and colorectal cancer [9-12]. Besides, VPA inhibits HCC cell proliferation and induces apoptosis via inducing autophagy, enhancing reactive oxygen species (ROS) generation, accompanied by the suppression of Akt signaling [13, 14]. Our previous results showed that VPA inhibited Notch signaling and antagonized Notch1-stimulated cell proliferation in vitro, indicating that VPA was a promising

reagent in treating HCC [15]. Notch signaling pathway, consisting of 4 transmembrane receptors (Notch1-4) and 5 transmembrane ligands (Jagged1-2, Delta1, 3, 4), plays a pivotal role in development, homeostasis, tumorigenesis of liver [16, 17]. Activation of Notch signaling requires a binding between the receptors and corresponding ligands within neighboring cells. Abnormal expression of Notch will lead to many liver diseases like Alagille syndrome, fatty liver and liver cancer [18, 19]. Evidence has shown that aberrant accumulation of Notch signaling was observed in human hepatocellular carcinoma samples [20, 21] and constitutive activation of Notch1 or Notch2 induces HCC formation in mice [22, 23]. Thus, selectively targeting Notch signaling with specific antibodies or inhibitors offers a new therapeutic strategy for liver disease, including HCC [19, 24-26]. It was reported that VPA acted synergistically with gossypol and aspirin in promoting the apoptosis of HCC cells [27, 28], providing us with an idea that combination of sorafenib with VPA may improve treatment efficacy of sorafenib. Herein, we investigated the anti-tumor activity of sorafenib combined with VPA in HCC both in vitro and in vivo. We demonstrated that combination of sorafenib and VPA not only inhibited HCC cells proliferation, promoted apoptosis synergistically in vitro, but also significantly decreased tumor volume compared to sorafenib or VPA alone in a nude mice subcutaneous xenograft model. Besides, VPA inhibited sorafenib-induced Notch3 augmentation and Akt phosphorylation in HCC cells. These findings raise the possibility of combination of sorafenib and VPA for HCC therapy.

Materials and methods

Cells and reagents

The human HCC cell lines (SK-Hep1, HepG2) were ordered from cell bank of Chinese Academy of Science (Shanghai, China). Cells were grown in DMEM medium (Hyclone) supplied with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and cultured in humidified incubator with 5% CO_2 at 37°C. Sorafenib tosylate was purchased from MedChemExpress (USA). Valproic acid sodium (VPA) was from Sigma-Aldrich (China). Cell counting kit-8 (CCK-8) was from Beyotime (China). Annexin V-FITC apoptosis detection kit was from BD Pharmingen (USA). The primary antibodies against Notch1 (#4380), Notch2 (#5732), Notch3 (#5276), Phospho-Akt (Ser-473, #4060), Akt (#4691), p21 (#2947), Bax (#2772), caspase9 (#9502) were from Cell Signaling Technology (USA). The antibodies against β-actin (sc-47778), Notch4 (sc-5594), Bcl-xL (sc-136132) were from Santa Cruz Biotechnology (USA). PARP1 (66520-1-Ig), caspase3 (66470-2-Ig) were from Proteintech (China).

Cell viability assay

Cell viability was measured by CCK-8 assay according to the manufacture's instruction. Briefly, cells were grown to 80% confluence, trypsinized, seeded at a density of 3000 cells per well in 96 well plates, incubated overnight and treated with various concentration of Sorafenib (in dimethyl sulfoxide, DMSO), VPA (in DMSO), or Sorafenib + VPA for another 72 hours. Then, medium was replaced with 100 μl fresh medium containing 10 μl CCK-8 solution and incubated at 37°C for 1 hour. Optical Density (OD) value at 450 nm was determined on microplate reader (Tecan, Switzerland). Cell viability was calculated by the equation: cell viability (%) = $(OD_{drug}-OD_{blank})/(OD_{DMSO}-OD_{blank}) \times 100\%$. The IC_{50} value was calculated by GraphPad Prism 5 software. Assessment of synergy was conducted by Compusyn software version 1.0. Combined index (CI) value less than one was considered as synergistic.

Cell apoptosis analysis

Assessment of cell apoptosis was measured by Annexin V-FITC apoptosis detection kit following the manufacturer's direction. Cells were placed in 6 well plates at around 2×10^5 cells per well, overnight, treated with certain concentration of Sorafenib, VPA, or Sorafenib plus VPA for 72 h. And then, cells were collected, washed twice with cold phosphate buffer solution (PBS) and re-suspended with 500 μl 1 × Annexin V binding buffer. After staining with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) for 15 minutes at room temperature in dark place, cells were analysed by flow cytometer (FlowSight amnis, Merck Millipore). Cells with Annexin V+/PI- and Annexin V+/PI+ staining were considered as apoptotic.

Protein extraction and preparation

Cells were placed on six-well plate and treated with various concentration of Sorafenib, VPA, or both for 72 h. Then, cells were harvested, washed with cold PBS and lysed with RIPA buffer (Beyotime) containing protease inhibitor (Sigma), Phenylmethanesulfonyl fluoride (PMSF, Sigma) and phosphatase inhibitor (Roche) on ice. Lysate was centrifuged at 12,000 g for 10 minutes at 4°C after sonicated. The total protein quantity was measured by bicinchoninic acid (BCA) protein assay kit (Beyotime) and boiled with 5 × loading buffer (Sangon, China) for 10 minutes at 98°C. After cooling on ice, samples were frozen and stored at -80°C.

Western blot analysis

Samples containing equal amount of protein were subjected to 8%, 10%, or 12% SDS-PAGE electrophoresis and transferred on polyvinylidene fluoride (PVDF) membrane (Millipore). After blocked in 5% non-fat milk (in tris-buffered solution with 0.1% tween 20, TBST) for 2 h at room temperature, the membrane was incubated with indicated primary antibody at 4°C overnight. Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (1:5000) was subsequently used as second antibody. Protein bands were developed with the chemiluminescence substrate (Sharebio, China). Optical density of each band was determined by Quantity One imaging software (Bio-Rad).

In vivo tumor xenograft model

All animal studies were approved by Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University and The Fifth People's Hospital of Shanghai Affiliated to Fudan University. Five-week-old male nude mice were from Shanghai SLAC Laboratory Animal Co.Ltd. and maintained under specific-pathogen-free facilities. Mice were subcutaneously injected with SK-Hep1 cells (5×10^6 in 100 μL PBS) and divided into 4 groups: control group, Sorafenib group (20 mg/kg), VPA group (200 mg/kg), Sorafenib (20 mg/kg) + VPA (200 mg/kg) group (n = 6 per group), when the tumor reached around 100 mm^3. Sorafenib was dissolved in solvent (cremophor EL/ethanol, 50%:50%) as 4 × stock solution, wrapped in aluminum foil and stored at room temperature. Stock solution was diluted 1:3 by water to 1 × solution when used. VPA sodium was dissolved in water, foil wrapped and stored at 4°C. Both sorafenib and VPA were given by gavage and prepared fresh every 2 days. Tumor volume (V) was measured every 2 days by caliper and calculated by the formula: $V = 0.5 \times L \times W^2$; L, W refers to the length and width of tumor respectively. After 21 days' treatment, mice were given anesthesia and blood was collected. Tumor nodules were immediately removed, weighted and fixed in 10% formaldehyde solution or frozen in liquid nitrogen. Protein in tumor nodule was extracted by homogenate method, and prepared as described above.

Serum analysis

The blood of mice was collected, resting for 30 minutes at room temperature and centrifuged at 3000 g for 10 minutes at 4°C. Serum was isolated and the activity of alanine aminotransferase (ALT) and content of blood urea nitrogen (BUN) was determined by Cobas 8000 modular analyzer series (Roche).

Histopathology analysis

The formalin-fixed and paraffin-embedded mice tissues were sliced into 4-μm-thick sections. After dewaxing and hydration, sections were stained with hematoxylin and eosin (HE). All images were filmed at 100 × or 200 × magnification.

Statistical analysis

Each experiment was conducted independently at least three times. Date were represented as means ± SD. Statistical comparisons were analyzed by the Students' t-test or one-way ANOVA via SPSS Statistics 19.0 software. *P* value < 0.05 was considered statistically significant.

Results

VPA and sorafenib inhibit HCC cell proliferation synergistically in vitro

Human hepatocellular carcinoma (HCC) cell lines SK-Hep1 and HepG2 were used to assess the anti-proliferation activity of VPA and sorafenib via CCK-8 assay. Both VPA and sorafenib effectively inhibited the viability of HCC cells in a dose-dependent manner. The IC_{50}

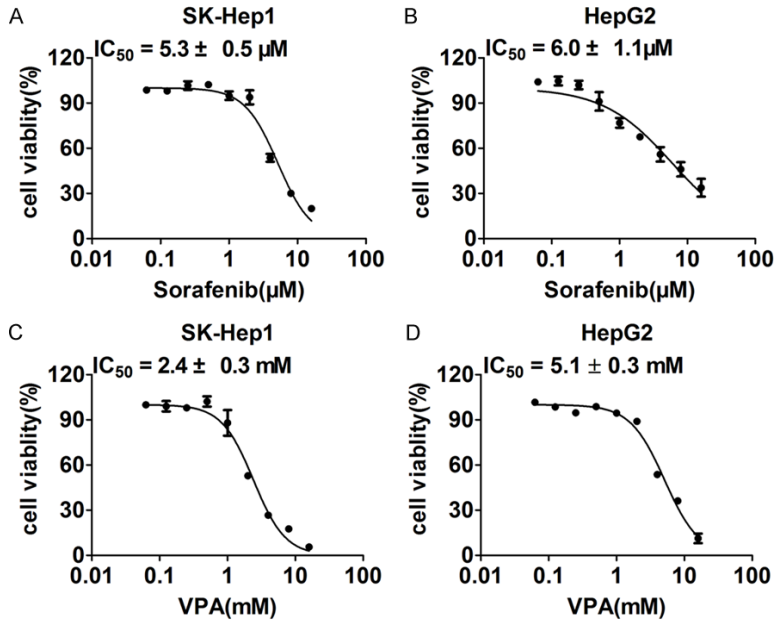

Figure 1. VPA inhibits HCC cells proliferation in vitro. SK-Hep1 and HepG2 cells were treated with a series of concentrations of sorafenib (A, B) or VPA (C, D) for 72 h, cell viability was measured by CCK-8 assay and IC_{50} value was calculated by GraphPad Prism 5.0 software. Data were represented by means ± SD from 3 independent experiments.

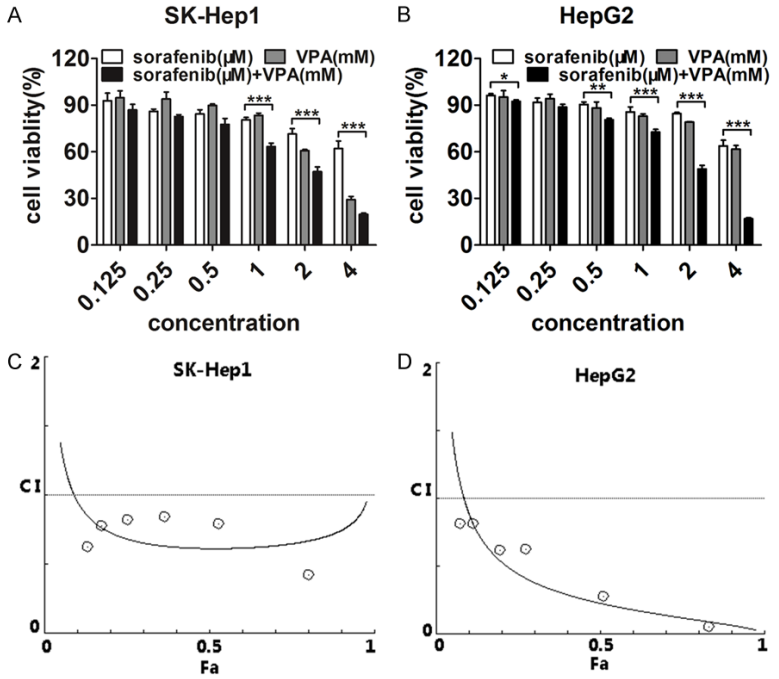

Figure 2. VPA and sorafenib inhibit HCC cell proliferation synergistically in vitro. A, B: SK-Hep1 or HepG2 cells were incubated with indicated concentrations of sorafenib (0.125, 0.25, 0.5, 1, 2, 4 μM; white column), VPA (0.125, 0.25, 0.5, 1, 2, 4 mM; gray column), or combination of both reagents (black column) in a constant molar concentration ratio (sorafenib : VPA = 1:1000) for 72 h. Cell viability was analyzed by CCK-8 assay. Data were shown by means ± SD from 3 independent experiments. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$. C, D: Combination Index (CI) was calculated by CompuSyn software 1.0, a CI value less than one was considered synergistic.

value of sorafenib is 5.3 ± 0.5 μM in SK-Hep1 and 6.0 ± 1.1 μM in HepG2 respectively. Moreover, the IC_{50} value of VPA is 2.4 ± 0.3 mM in SK-Hep1 and 5.1 ± 0.3 mM in HepG2 respectively (**Figure 1A-D**). A series of different concentrations of sorafenib (0.125, 0.25, 0.5 1.0, 2.0, 4.0 μM), VPA (0.125, 0.25, 0.5 1.0, 2.0, 4.0 mM) or combination of both reagents were investigated to identify whether sorafenib and VPA has synergistic effect on HCC. Cell viability showed a marked reduction when sorafenib was combined with VPA in a constant molar ratio (sorafenib : VPA = 1:1000) compared to sorafenib or VPA alone. In HepG2, cell viability decreased 47% ($P < 0.001$) when cells were treated with 4 μM sorafenib plus 4 mM VPA compared to that treated with 4 μM sorafenib alone. Similar results were also observed in SK-Hep1 (**Figure 2A**, **2B**). Combination index (CI) was calculated by the Chou-Talalay method [29] (**Figure 2C**, **2D**). CI value less than one indicated that this kind of combination is synergistic in both tested cells. These results suggest that VPA inhibits HCC cell proliferation and acts synergistically with sorafenib in vitro.

VPA enhances pro-apoptotic effect of sorafenib in HCC cells

To determine whether VPA could increase sorafenib-induced cell apoptosis, SK-Hep1 and HepG2 cells were treated with DMSO, sorafenib, VPA or sorafenib plus VPA for 72 h and then analyzed by flow cytometry. Sorafenib plus VPA induced sig-

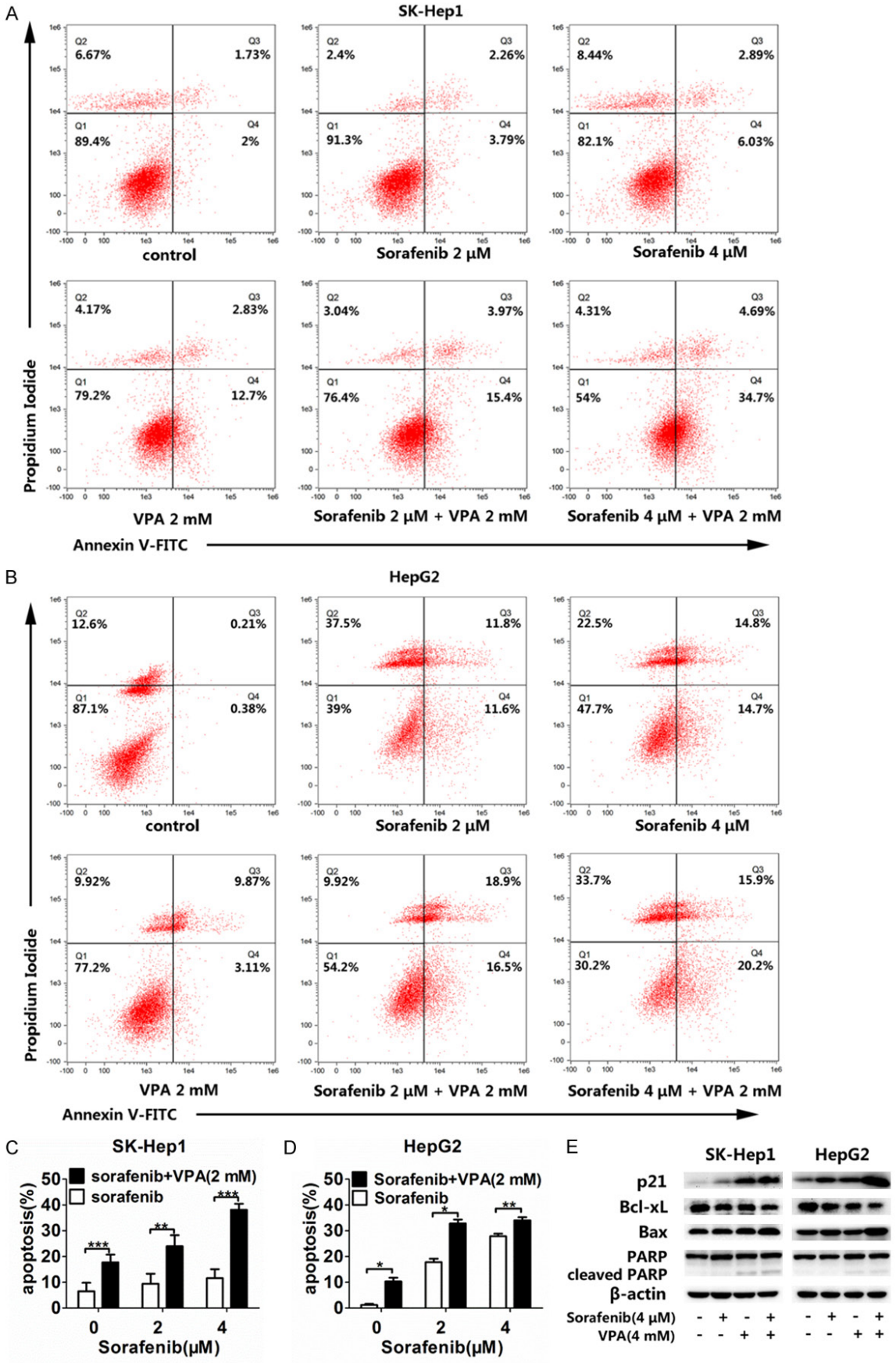
A
SK-Hep1
Q2 6.67%
Q3 1.73%
Q1 89.4%
Q4 2%
control
Q2 2.4%
Q3 2.26%
Q1 91.3%
Q4 3.79%
Sorafenib 2 μM
Q2 8.44%
Q3 2.89%
Q1 82.1%
Q4 6.03%
Sorafenib 4 μM
Q2 4.17%
Q3 2.83%
Q1 79.2%
Q4 12.7%
VPA 2 mM
Q2 3.04%
Q3 3.97%
Q1 76.4%
Q4 15.4%
Sorafenib 2 μM + VPA 2 mM
Q2 4.31%
Q3 4.69%
Q1 54%
Q4 34.7%
Sorafenib 4 μM + VPA 2 mM
Propidium Iodide
Annexin V-FITC
B
HepG2
Q2 12.6%
Q3 0.21%
Q1 87.1%
Q4 0.38%
control
Q2 37.5%
Q3 11.8%
Q1 39%
Q4 11.6%
Sorafenib 2 μM
Q2 22.5%
Q3 14.8%
Q1 47.7%
Q4 14.7%
Sorafenib 4 μM
Q2 9.92%
Q3 9.87%
Q1 77.2%
Q4 3.11%
VPA 2 mM
Q2 9.92%
Q3 18.9%
Q1 54.2%
Q4 16.5%
Sorafenib 2 μM + VPA 2 mM
Q2 33.7%
Q3 15.9%
Q1 30.2%
Q4 20.2%
Sorafenib 4 μM + VPA 2 mM
Propidium Iodide
Annexin V-FITC
C
SK-Hep1
sorafenib+VPA(2 mM)
sorafenib
apoptosis(%)
Sorafenib(μM)
D
HepG2
Sorafenib+VPA(2 mM)
Sorafenib
apoptosis(%)
Sorafenib(μM)
E
SK-Hep1
HepG2
p21
Bcl-xL
Bax
PARP
cleaved PARP
β-actin
Sorafenib(4 μM)
VPA(4 mM)

Figure 3. VPA combined with sorafenib significantly induces apoptosis in HCC cells. A, B: Cells were exposed to indicated dosage of sorafenib, and/or 2 mM VPA for 72 h, stained with Annexin-V FITC and PI, then analyzed by flow cytometry. C, D: The percentage of apoptosis (%) (FITC+/PI- and FITC+/PI+) was calculated. E: Whole cell lysates were collected after 72 h of treatment of indicated drugs and subjected to western blot analysis. Representative bands were shown. Data were represented by means ± SD from 3 independent experiments. *, P < 0.05, **, P < 0.01 and ***, P < 0.001.

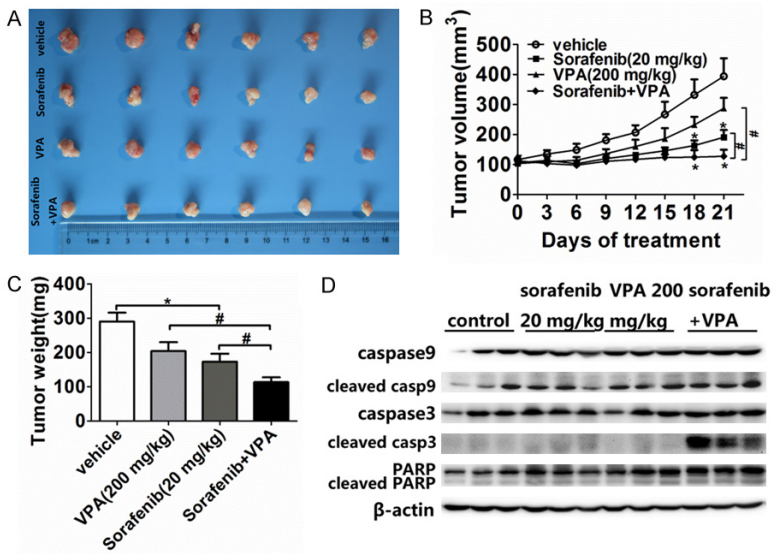

Figure 4. VPA potentiates anti-tumor effects of sorafenib in vivo. (A, B) Mice bearing SK-Hep1 subcutaneous xenograft were divided into 4 groups and given indicated treatment every day (vehicle, sorafenib 20 mg/kg, VPA 200 mg/kg, sorafenib 20 mg/kg + VPA 200 mg/kg). Tumor volumes were measured every 2 days. Tumors were removed from mice after 21 days treatment (A) and weighted (C). Data were shown by means ± SD (n = 6). *, P < 0.05, compared to vehicle group; #, P < 0.05 compared to combination group. (D) The expression of cleaved caspase9, cleaved caspase3, cleaved PARP from tumor tissue homogenates were analyzed by western blot.

nificantly apoptosis (P < 0.05) compared to corresponding single treatment in both cells (**Figure 3A**, **3B**). Detailedly, the percentage of apoptosis in combination group was increased by 11.2%, 14.5%, 26.5% in SK-Hep1 (P < 0.01) and 9.1%, 15%, 6.2% in HepG2 (P < 0.05) than sorafenib group respectively at indicated dosage (**Figure 3C**, **3D**). Moreover, these results were confirmed by western blot that sorafenib plus VPA remarkably increased the level of cell cycle inhibitor p21, pro-apoptotic protein Bax, cleaved PARP and decreased anti-apoptotic protein Bcl-xL compared to mono-therapy group (**Figure 3E**). These findings demonstrate that VPA enhances the pro-apoptotic ability of sorafenib in HCC cells.

VPA potentiates anti-tumor effects of sorafenib in vivo

To evaluate the anti-tumor efficacy of combination of sorafenib and VPA in vivo, nude mice bearing SK-Hep1 subcutaneous xenograft model were established. Vehicle (cremophor EL:ethanol:water = 12.5%:12.5%:75%), VPA (200 mg/kg, in H_2O), sorafenib (20 mg/kg, in vehicle) or combination (VPA 200 mg/kg + sorafenib 20 mg/kg) was given by gavage every day when the tumor volume reached around 100 mm^3. Compared to sorafenib group, the combination group showed a significant decrease in tumor volume (287 ± 84 mm^3 vs. 127 ± 53 mm^3, P < 0.05) (**Figure 4A**, **4B**). Twenty-one days after indicated drugs administration, the average tumor weight of combination group vs. sorafenib group is 113.4 ± 31.2 mg vs. 173.2 ± 52 mg respectively (around 35% inhibitory rate, P < 0.05) (**Figure 4C**). Western blot analysis of tumor tissue protein was conducted to examine the level of apoptosis-associated protein. Cleaved caspase9, cleaved caspase3 and cleaved PARP were increased markedly in the combination group compared to either control or mono-therapy group, indicating that combination of sorafenib and VPA could remarkably activate the intrinsic apoptotic pathway (**Figure 4D**). These findings suggest that VPA improves the anti-tumor effects of sorafenib in vivo.

Safety evaluation of VPA plus sorafenib in vivo

After twenty-one days indicated treatment, histological analysis of liver and kidney paraffin section from the four groups of mice exhibited well-preserved structure and no inflammatory cell infiltration (**Figure 5A**). VPA showed quite good tolerance as there was no significant difference in the average body weight between VPA and control group (26.7 ± 0.6 vs. 26.2 ± 1.8 g, P = 0.483) at the dosage of 200 mg/kg.

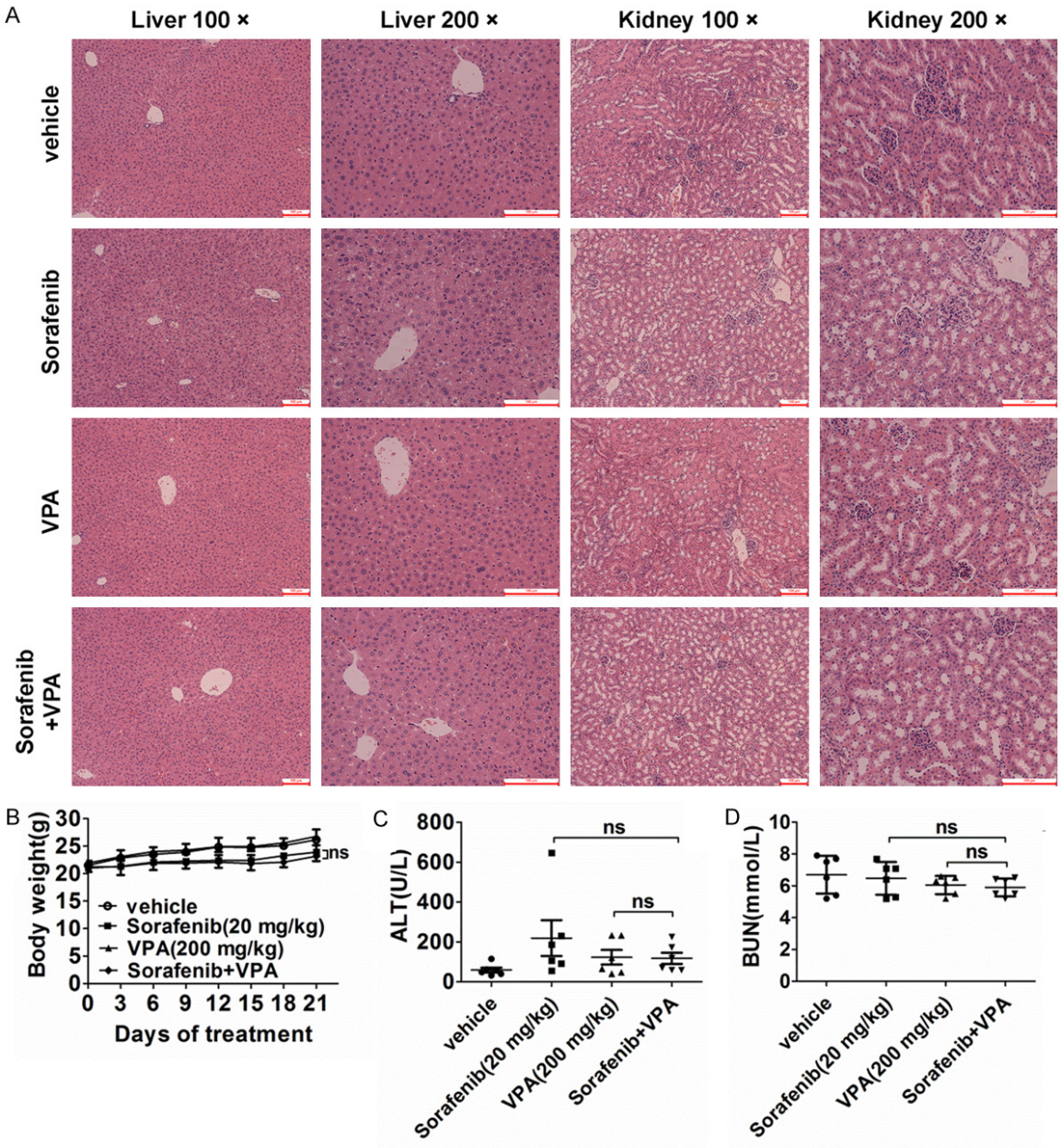

Figure 5. Safety evaluation of VPA combined with sorafenib in vivo. A: Liver and kidney paraffin sections from mice receiving indicated drugs or vehicle alone were stained with hematoxylin & eosin. Representative sections were photographed at the magnification of 100 × or 200 ×. Scale bars = 100 μm. B: Body weight of mice during the treatment. C, D: ALT and BUN level in the serum after 21 days treatment. Data were shown by means ± SD with n = 6 per group. (ns means no significance).

Moreover, combination group showed similar average body weight to that of sorafenib group (23.3 ± 0.8 vs. 23.9 ± 1.2 g, P = 0.254), suggesting that sorafenib plus VPA had no effect on normal growth compared to sorafenib alone (**Figure 5B**). To further evaluate the safety of this combination formula, we measured the serum level of alanine aminotransferase (ALT) and urea nitrogen (BUN), which are serological indicator of liver and kidney function respectively. There was no significant difference in the level of serum ALT or BUN between combination group and mono-therapy group (**Figure 5C**, **5D**). In addition, there was no lethality in any

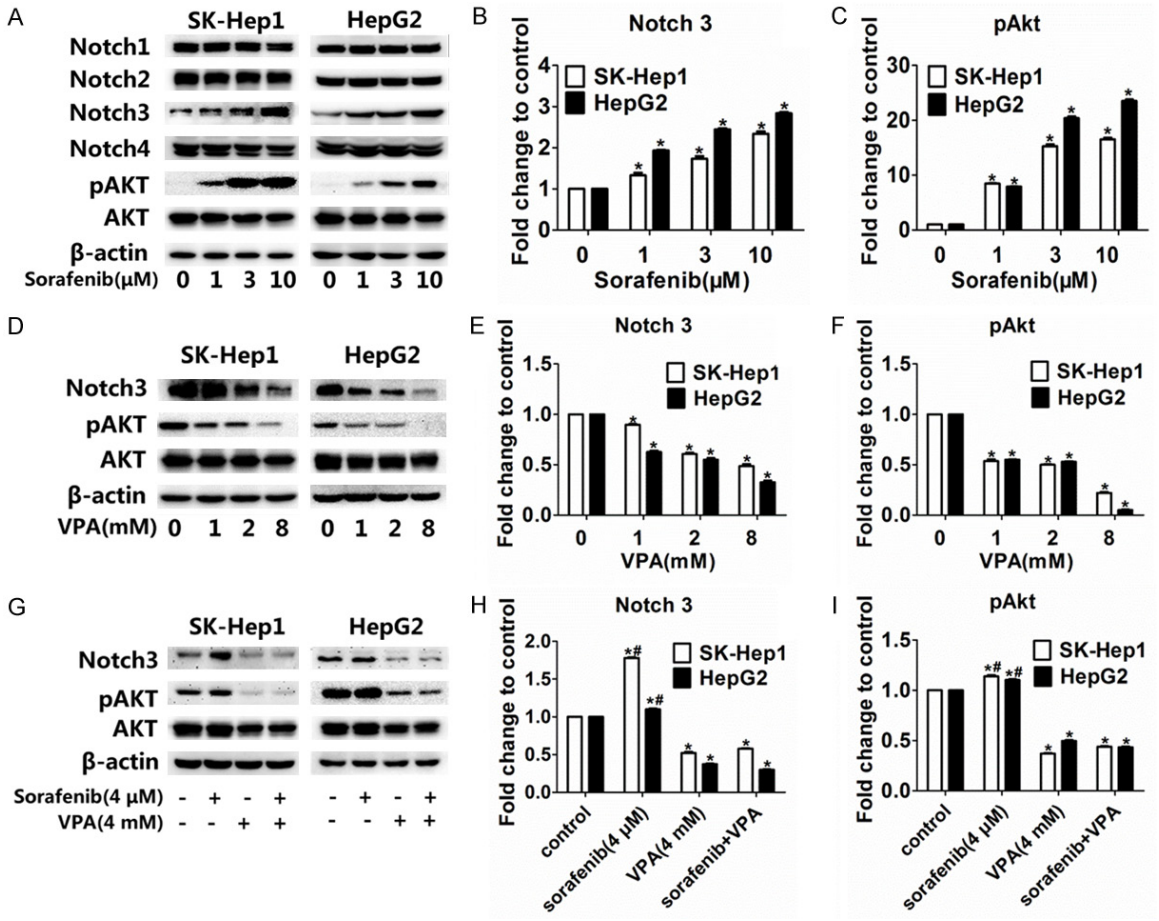

Figure 6. VPA down-regulates sorafenib-induced Notch3 and pAkt in HCC. SK-Hep1 or HepG2 cells were treated with indicated concentration of sorafenib (A-C), VPA (D-F), or sorafenib and/or VPA (G-I) for 72 h, whole cell lysates were detected by antibodies specific to Notch1, Notch2, Notch3, Notch4, pAkt and Akt via western blot assay. The intensity value of bands were analyzed by Quantity-One software and expressed as fold change compared to control. Representative bands were shown in (A, D, G) and quantification of data were analyzed and represented in (B, C, E, F, H, I). Data were shown as means ± SD from three independent experiments. *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to combination group.

treatment group. Altogether, these results suggest that combination of VPA and sorafenib exhibits well tolerance in vivo.

VPA down-regulates sorafenib-induced Notch3 and pAkt in HCC

Our previous results showed that VPA could induce HCC cells growth arrest via suppressing Notch signaling. Therefore, we compared the expression levels of four Notch receptors to explore the role of Notch in the synergistic effect of sorafenib and VPA. Sorafenib significantly increased the expression of Notch3 in a dose-dependent manner. In comparison, the up-regulation of Notch1, Notch2 and Notch4 was less pronounced. Besides, sorafenib also led to a dose-dependent increase of Akt phosphorylation in HCC cells (**Figure 6A-C**), which compromised by VPA (**Figure 6D-F**). The induction of Notch3 and pAkt by sorafenib was significantly attenuated when VPA was combined with sorafenib (**Figure 6G-I**). Collectively, these findings suggest that VPA synergistically potentiates anti-hepatoma efficacy of sorafenib might through decreasing Notch3 expression and Akt phosphorylation.

Discussion

It is acknowledged that the inhibition of one canonical signaling will activate another compensatory pathway in the treatment of cancer, underlying the potential mechanism of resis-

tance [7, 30]. Therefore, combination therapies applying agents aiming at different targets is prevalent in cancer managements. Hepatocellular carcinoma is resistant to almost every single chemotherapy drug [31, 32]. Sorafenib remains the standard systemic treatment for advanced hepatocellular carcinoma, blocking multiple kinases while stimulating Akt phosphorylation [33]. Attempts to combined sorafenib and Akt signaling inhibitor have been made that demonstrated better anti-tumor effects than sorafenib alone in HCC [34, 35]. Previously, we have reported that VPA suppressed Notch signaling in hepatoma cell SK-Hep1 and reversed Notch1-stimulated cell proliferation. In the current study, we explored the preliminary efficacy of reasonably-designed combination of sorafenib and VPA in the treatment of HCC both in vitro and in vivo. Cell viability was decreased markedly and the percentage of apoptotic cells increased significantly in the combination group compared to sorafenib alone, suggesting that sorafenib and VPA worked in a synergistic manner, which was further validated by the data of combination index. Moreover, the up-regulation of cell cycle progression inhibitor $p21^{Waf1/Cip1}$ in combination group indicated VPA and sorafenib co-treatment may induce cell cycle arrest. The reduction of anti-apoptotic protein Bcl-xL and elevation of pro-apoptotic protein Bax, cleaved caspase9, cleaved caspase3 and cleaved PARP manifested the activation of intrinsic apoptotic pathway in the combination of VPA and sorafenib. Besides, the combination of VPA and sorafenib down-regulated the effective dosage of sorafenib in treating HCC, which may decrease the dosage-associated side effects of sorafenib. Using a subcutaneous xenograft model in nude mice, we examined the anti-hepatoma efficacy of combination of VPA and sorafenib in vivo. Mice with combined sorafenib and VPA administration showed decreased tumor volume and weight compared to sorafenib or VPA group. Histological and serological examinations exhibited well tolerance of this combination. Altogether, these findings strongly support therapeutic potential of sorafenib in combination with VPA for HCC.

Studies of combination of sorafenib and other targeting or chemotherapeutic reagents to improve the managements of HCC have been extensively made since the approval of sorafenib in advanced HCC, by the means of avoiding activation of alternative pro-survival signaling or drug resistance [36, 37]. In this study, for the first time to our knowledge, our data showed that sorafenib induced upregulation of Notch3 in a concentration dependent manner, along with the activation of Akt. The role of Notch signaling in cancer is highly context and subtype dependent, among which Notch3 plays an important role in the chemotherapeutic resistance in HCC [38]. It was confirmed via specific shRNA that inhibition of Notch3 improved the anti-tumor efficacy of doxorubicin and sorafenib [26, 39]. It was also reported that deregulated PI3K/Akt/mTOR signaling pathway plays an important role in the prognosis of HCC [33]. Akt phosphorylation induces numerous downstream signaling that promotes cell survival, proliferation and metabolism [40, 41]. As a result, the activation of Akt survival signaling might be a responsive reply of HCC cells to sorafenib-induced stimuli, which helps cells survive, thus compromising the anti-HCC effect of sorafenib [33, 42, 43]. There are reports about the positive relationship between Notch1 and Akt in HCC that cell viability was decreased drastically with the reduction in Notch1 expression after treatment of Akt inhibitor. And vice versa, knockdown of Notch1 via specific shRNA reduced the Akt phosphorylation [44]. However, it is unclear whether Notch3 inhibition alone will inhibit Akt activation, which needs further detailed study and confirmation. We discovered that VPA inhibited Notch3 expression in a concentration dependent manner. Likewise, Akt phosphorylation reduction was also observed in the treatment of VPA. Combined sorafenib and VPA could antagonize sorafenib-induced overexpression of Notch3 and Akt phosphorylation. Based on these results as well as the current understanding of Notch signaling, we postulate that Notch3 and Akt are responsible for the resistance of sorafenib in HCC and VPA may overcome the resistance to improve the anti-HCC efficacy. Further investigation into the exact role for Notch3 and Akt may facilitate the clarification of resistance of sorafenib in HCC. The combination of VPA and sorafenib in preclinical studies paves the way for further detailed examination, which offers a promising strategy for HCC treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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