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Synergistically killing activity of aspirin and histone deacetylase inhibitor valproic acid (VPA) on hepatocellular cancer cells

Xiaofei Li [a], Yanshuang Zhu [a], Huabin He [b], Lianqing Lou [a], Weiwei Ye [a], Yongxin Chen [a], Jinghe Wang [a,*]

[a] Department of Infectious Diseases, Yiwu Central Hospita, 519 Nan men Street, Yiwu, Jinhua, Zhejing 322000, China
[b] Department of Orthopedics, Yiwu Central Hospita, 519 Nan men Street, Yiwu, Jinhua, Zhejing 322000, China

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ABSTRACT

Aspirin and valproic acid (VPA) have been extensively studied for inducing various malignancies growth inhibition respectively, despite their severe side effects. Here, we developed a novel combination by aspirin and VPA on hepatocellular cancer cells (HCCs). The viability of HCC lines were analyzed by MTT assay, apoptotic analysis of HepG2 and SMMC-7721 cell was performed. Real time-PCR and Western blotting were performed to determine the expression of apoptosis related genes and proteins such as Survivin, Bcl-2/Bax, Cyclin D1 and p15. Moreover, orthotopic xenograft tumors were challenged in nude mice to establish murine model, and then therapeutic effect was analyzed after drug combination therapy. The viability of HCC lines' significantly decreased after drug combination treatment, and cancer cell apoptosis in combination group increasingly induced compared with single drug use. Therapeutic effect was significantly enhanced by combination therapy in tumor volume and tumor weight decrease. From the data shown here, aspirin and VPA combination have a synergistic killing effect on hepatocellular cancers cells proliferation and apoptosis.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer in parts of Africa and Asia, often occurs more in men than women, and usually seen in people age 50 or older. Lots of epidemiological evidence shown that clear risk factors such as chronic alcohol intake, aflatoxin exposure, and cirrhosis of any etiology play a critical role in HCC development besides of viral infections (HBV or HCV) [1]. Like many other cancers, early symptoms in this disease are extremely difficult to make early diagnosis, and few patients with HCC are suitable for surgical resection at the time of diagnosis. Meaning while, intense investigation on molecular pathogenesis of HCC needs to be carried out to identify new targets to prevent or treat this global health problem. However, conventional chemotherapy does not provide significant clinical efficiency or prolonged survival for advanced HCC patients. Therefore, it is necessary to identify novel therapeutic strategies for the treatment of HCC.

Non-steroidal anti-inflammatory drug (NSAID) aspirin (acetylsalicylic acid) could inhibit the activity of the cyclooxygenase enzymes (COX-1 and COX-2), has been linked to anti-oxidative, anti-microbial, anti-inflammatory [2–8]. Newly report shown that aspirin has additional antitumor activity in several cancer cell lines, and has been shown to reduce the risk associated with various types of malignancies [9,10]. Therefore, aspirin will be considered as a promising candidate for cancer prevention in future.

Another old and widely used drug, valproic acid (VPA), a potent and specific inhibitor of HDAC, is also known to induce growth inhibition and/or apoptosis in a broad spectrum of cancer cells [11,12]. Its anti-tumor effect is considered to occur primarily through the increase histone acetylation and active apoptosis related gene expression, but the underlying mechanisms are still not clearly elucidated. Mesothelioma cells growth suppression and apoptosis could be induced by VPA in recent studies [13,14].

However, whether aspirin combined with VPA has a synergistic effect on hepatocellular cancer cell lines' proliferation and apoptosis is still unkown. Based on these findings, in the present study, we aim to evaluate the synergistic anti-tumor effect of aspirin and VPA combination in human hepatocellular cancer cell lines.

2. Materials and methods

2.1. Cell lines, cell culture and reagents

Human HCC lines (Huh7, HepG2, SMMC-7721, Hep3B and H22) and normal hepatocellular cell line LO2 were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured immediately after the thawing of the cells at a concentration of 5×10^6 cells/mL in RPMI 1640 culture medium (Hyclone, USA)

* Corresponding author.
E-mail address: Xiaofeili2000@163.com (J. Wang).

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supplemented with 10% FBS (Hyclone, USA), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were incubated in a humidified atmosphere containing 5% carbon dioxide at 37 °C. Fluo-3/AM, aspirin and VPA (Sigma, USA) were kept as a stock solution (100 mmol/L) in PBS, and then diluted to the appropriate working concentration.

2.2. Cell viability assay

The viability of HepG2, SMMC-7721 cells after aspirin, VPA and their combination treatment were determined by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 4.0×10^3 cells per well (100 μL), and incubated for 24 h till all cells sufficient attached to the plate. Before the drug treatment, the culture medium changed as RPMI 1640 medium contained 2% FBS. After 24 h, 48 h, 72 h or 96 h treatment with varying doses of single drug or its combination. After treatments, medium were discarded, washed with PBS and incubated with MTT (5 mg/mL in phosphate-buffered saline) in 100 μL fresh medium, incubated at 37 °C for another 4 h. Discard the medium, 200 μL DMSO was added to dissolve MTT formazan crystals and absorbance at 570 nm was measured using a multiwell plate reader (BioTek, Winooski, VT, USA). Wells containing only RPMI1640 and MTT were used as negative control (NC). Cell viability was calculated as percentage of viable cells in total population. Each experiment was performed three replicates.

2.3. Invasion assays and motility assays

Assays were performed by a modification of the method described by Albini and colleagues [15,16]. For invasion assay, filters were coated with Matrigel™ (Sigma) diluted to 1 mg/mL in serum-free RPMI1640 medium. The inserts were incubated at 37 °C for 1 h to allow gel polymerisation. HepG2 and SMMC-7721 cells were harvested and suspended in RPMI containing 10% FBS at a concentration of 1×10^6 cells/mL. Cells were incubated at 37 °C for 24 h. Afterwards, the inner side of the filter was wiped with a wet swab to remove the cells while the outer side of the insert was rinsed with PBS and stained with 0.25% crystal violet (Sigma) for 10 min, rinsed again and then allowed to dry. The filters were then viewed under microscope and the percent of area occupied by migrated or invading cells was analyzed to determine the total number. The procedure for carrying out motility assays was identical to the procedure used for invasion assays with the exception that the inserts were not coated with matrigel.

2.4. Apoptosis assay

The Annexin V-PE detects phosphatidyl-serine on the external membrane of apoptotic cells and 7-AAD, a cell impermeant dye, is an indicator of membrane structural integrity. 7-AAD is excluded from live cells and early apoptotic cells, but permeates late-stage apoptotic and dead cells. Cells which stain positive for Annexin V-PE and negative for 7-AAD are classified as early apoptotic cells, whilst cells which are positive for both represent late apoptotic cells. The ability of the aspirin and VPA combination to induce apoptosis was assessed in HepG2 and SMMC-7721 cells. Briefly, 5×10^5 cells were seeded per well in 12 well plates and incubated overnight to allow attachment. Cells were treated with aspirin, VPA or in combination for 48 h. Cisplatin was used as a control for induction of apoptosis. After 48 h apoptotic cells in the medium were collected and adherent cells were trypsinized. The resulting pool of cells was centrifuged at 300*g* for 5 min, then resuspended in 50 μL of fresh medium and transferred to a round-bot-tom 96 well plate (Costar). 10 μL Annexin V-PE and 5 μL 7-AAD were added to each well and the plate was incubated protected from light for 20 min at room temperature. After incubation the samples were acquired on the Guava EasyCyte. Each experiment was repeated three times and reproducible results were obtained.

2.5. Mitochondrial membrane potential assays

The loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event preceding phosphatidylserine externalization and coinciding with caspase activation. MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA, USA) exhibits time-dependent accumulation in mitochondria, indicated by a shift in its fluorescence emission from green to red. Therefore, mitochondrial depolarization could be indicated by red/green fluorescence ratio. HepG2 and SMMC-7721 cells were harvested after a 24 h exposure to aspirin, VPA alone or in combination, and then centrifuged at 400*g* for 5 min; the cell pellet was resuspended in 0.1 μmol/L final concentration of MitoTracker Red CMXRos (dissolved in dimethyl sulfoxide) for 20 min. Cells were washed and resuspended in PBS, and then fixed with 4% paraformaldehyde. After another wash in PBS, the cells on the microplate were read by a spectrophotometer.

2.6. Clonogenic survival experiments

HepG2 and SMMC-7721 cells were seeded into 6-cm dishes. After 6 h, they were treated with concentrations of aspirin, VPA alone or in combination. One week later, colonies were fixed with methanol and stained with 1.25% Giemsa and 0.125% crystal violet for counting. Cell survival was expressed in relation to the untreated control. Values are given as mean of three independent experiments.

2.7. Determination of intracellular calcium concentration ($[Ca^{2+}]$)

The concentration of intracellular $Ca2^+$ was measured with Fluo-3/AM. After HepG2 and SMMC-7721 cells were incubated with aspirin, VPA alone or its combination for 24 h, the supernatant in each well was removed and HepG2 and SMMC-7721 cells were washed twice with PBS, and then incubated with 5 mmol/L Fluo-3/AM for 30 min at 37 °C, then remove the supernatant of the plate. Wash three times with PBS to remove the non-specific staining. Then moderate PBS was added to cover the cells. Finally, pictures were taken by laser scanning confocal microscopy with excitation at 488 nm and emission at 525 nm. The total cells counts and total fluorescent intensity were calculated with software, and the mean fluorescent intensity of each group was calculated, respectively. The concentration of intracellular Ca^{2+} was expressed by the mean fluorescent intensity.

2.8. qPCR

Cells (HepG2) were seeded and cultured for 24 h, and treated with asprin, VPA alone or its combination. After 24 h, cells were harvested and total RNA was isolated using Trizol (TianGen, Beijing). RNA was reverse transcribed using a first-strand cDNA synthesis kit from Tiangen Corp (Beijing, China). RT-PCR was performed on an Applied Biosystems 7300 Real Time PCR system using SYBR Green incorporation following standard protocols. Ct values were calculated based on duplicates and normalized to the housekeeping gene β-actin. Primers were p15-F (5′-AAG CTG AGC CCA GGT CTC CTA-3′) and p15-R (5′-CCA CCG TTG GCC GTA AAC T-3′); Survivin-F (5′-AGA ACT GGC CCT TCT TGG AGG-3′) and Survivin-R (5′-CTT TTTA TGT TCC TCT ATG GGG TC-3′); DNMT3A-F (5′-CAG CGT CAC ACA GAA GCA TAT CC-3′) and DNMT3A-R (5′-GGT CCT CAC TTT GCT GAA CTT GG-3′); Hsp70-F (5′-CCT GAT CGG CCG CAA GTT CG-3′) and Hsp70-R (5′-TGC CCC CGC CCA GGT CAA AGA T-3′); Cyclin D1-F (5′-TGT TCG TGG CCT

CTA AGA TGA AG-3′) and Cyclin D1-R (5′-AGG TTC CAC TTG AGC TTG TTC AC-3′); Bcl-2 forward (5′-CAT GTG TGT GGA GAG CGT CAA-3′) and Bcl-2 reverse (5′-GCC GGT TCA GGT ACT CAG TCA-3′); Bax-F (5′-GCC CTT TTG CTT CAG GGT TT-3′) and Bax-R (5′-TCC AAT GTC CAG CCC ATG AT-3′); β-actin-F (5′-AGC GCA AGT ACT CCG TGT G-3′) and β-actin-R (5′-AAG CAA TGC TAT CAC CTC CC-3′).

2.9. Protein extraction and Western blotting

Briefly, cells plated at a density of 3×10^5/mL in 6-well plates were exposed to aspirin, VPA or a combination of the two drugs for 48 h prior to harvest. Following centrifugation and sonication, cell extracts were clarified at 12,000 rpm for 10 min at 4 °C. Protein concentrations were measured using a BCA assay. Protein samples (30 μg), diluted with SDS sample buffer, were separated by 10% polyacrylamide gel electrophoresis, and followed by electro-blotting on a polyvinylidene difuoride (PVDF) membrane. After blocking in 5% non-fat dry milk, the membrane was probed overnight at 4 °C with primary antibodies following: anti-β-actin, anti-Survivin, anti-Hsp70, anti-DNMT3A, anti-Bcl-2, anti-Bax, anti-p15, anti-Cyclin D1, immunoreactivity was detected using anti-mouse or anti-rabbit IgG conjugated peroxidase, then visualized by with the ECL kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.10. In vivo experiments

Mouse model was approved by the Institutional Animal Care and Use Committee, animal care was in strict compliance with the institutional guidelines.

BALB/c nude mice (5 weeks old) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy Sciences. HepG2 cells were grown, harvested, washed with PBS, and re-suspended in sterile, serum-free PBS. Each mice were injected subcutaneously (s.c.) into each posterior hind flank region with approximately 5.0×10^6 cells, one site per mouse. Xenografts were allowed to grow, and treatment was started when the injected cell mass reached a mean volume of 150 mm^3. After tumor formation, the mice were randomized into five groups ($N = 5$ per group), and administered aspirin (1 μg/kg/day, i.p.), VPA (500 mg/kg/day, i.p.), combination (1 μg/kg/day aspirin plus 500 mg/kg/day VPA, i.p.) and cisplatin (5 mg/kg/day, i.p.) for 1 week, saline only in the case of the untreated control group. Tumor growth was monitored twice weekly. The length (L) and the width (W) of the tumors were measured using a slide caliper and the volume (V) of each tumor was calculated as follows: $V = L \times W^2/2$.

2.11. Statistical analysis

All data were expressed as mean ± standard deviation (SD), significance was assessed using Student's t-test. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Aspirin and VPA inhibits the proliferation of hepatocellular cancer cell lines

To study the synergistic anti-proliferative effect of aspirin and VPA, drugs were treated separately with various concentrations for 12, 24, 48, 96 h. The cells were then analyzed by MTT method. As shown in Fig. 1, aspirin (Fig. 1A) and VPA (Fig. 1B) both significantly reduced viable HepG2 hepatocellular cancer cell numbers in a dose-dependent manner as revealed by the MTT assay ($P < 0.05$).

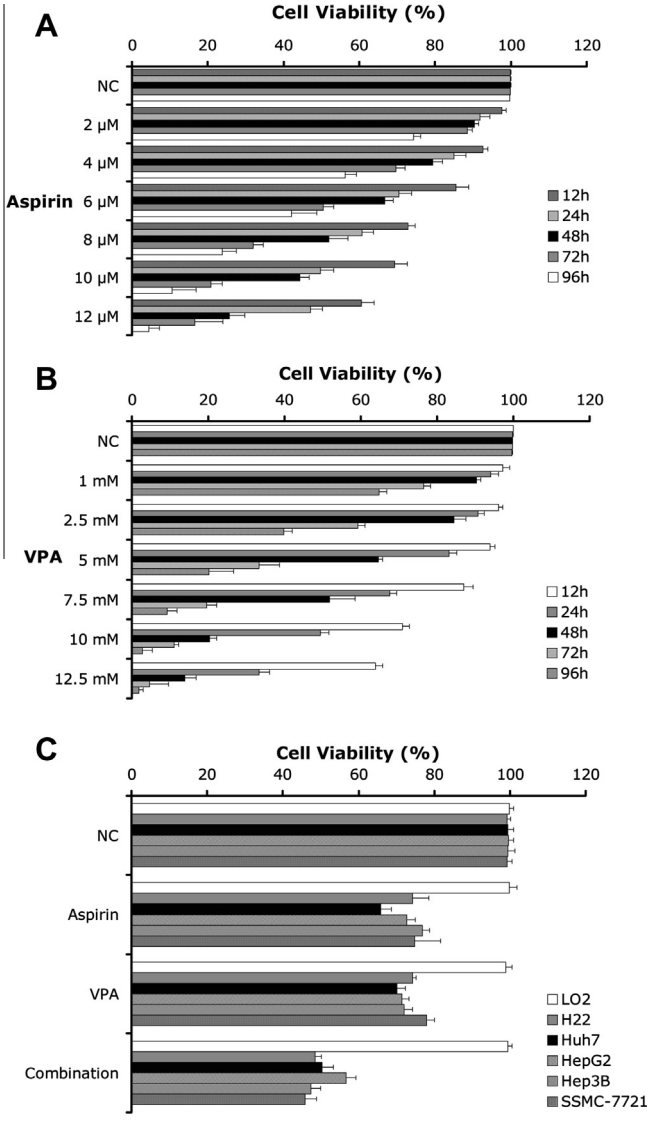

Fig. 1. Viability of aspirin and VPA on hepatocellular cancer cells. (A) Viability of different drug concentrations on HepG2 cells treated with aspirin, $P < 0.05$. (B) Viability of different drug concentration of VPA on HepG2 cells with different time treatment, $P < 0.05$. (C) Viability of combination group (4 μmol/L aspirin plus 2.5 mmol/L VPA) on normal hepatocellular cell line LO2 and different hepatocellular cancer cell lines (H22, Huh7, HepG2, Hep3B and SSMC-7721), $^*P < 0.05$. NC indicated as negative control.

Combined treatment with aspirin (4 μmol/L) and VPA (2.5 mmol/L) was then assessed at concentration of the drugs for 48 h, the combination of both agents exhibited a synergistic effect and induced an approximately 20% decrease in five hepatocellular cancer cell lines compared with drugs used separately ($P < 0.01$) (Fig. 1C). However, this synergistic effect does not exist in normal hepatocellular cell LO2.

3.2. Aspirin and VPA induce hepatocellular cancer cell lines apoptosis

The key feature of malignant cells is their capacity to move to distant sites, invade surrounding tissue, and its colony formation ability. The motility assays and invasion assays were performed. As is shown in Fig. 2A and B, combination treatment of 4 μmol/L aspirin and 2.5 mmol/L VPA suppressed HepG2 and SMMC-7721 cells' motility (Fig. 2A) and invasion (Fig. 2B) when compared to control or drugs used alone ($P < 0.05$), moreover, the combination

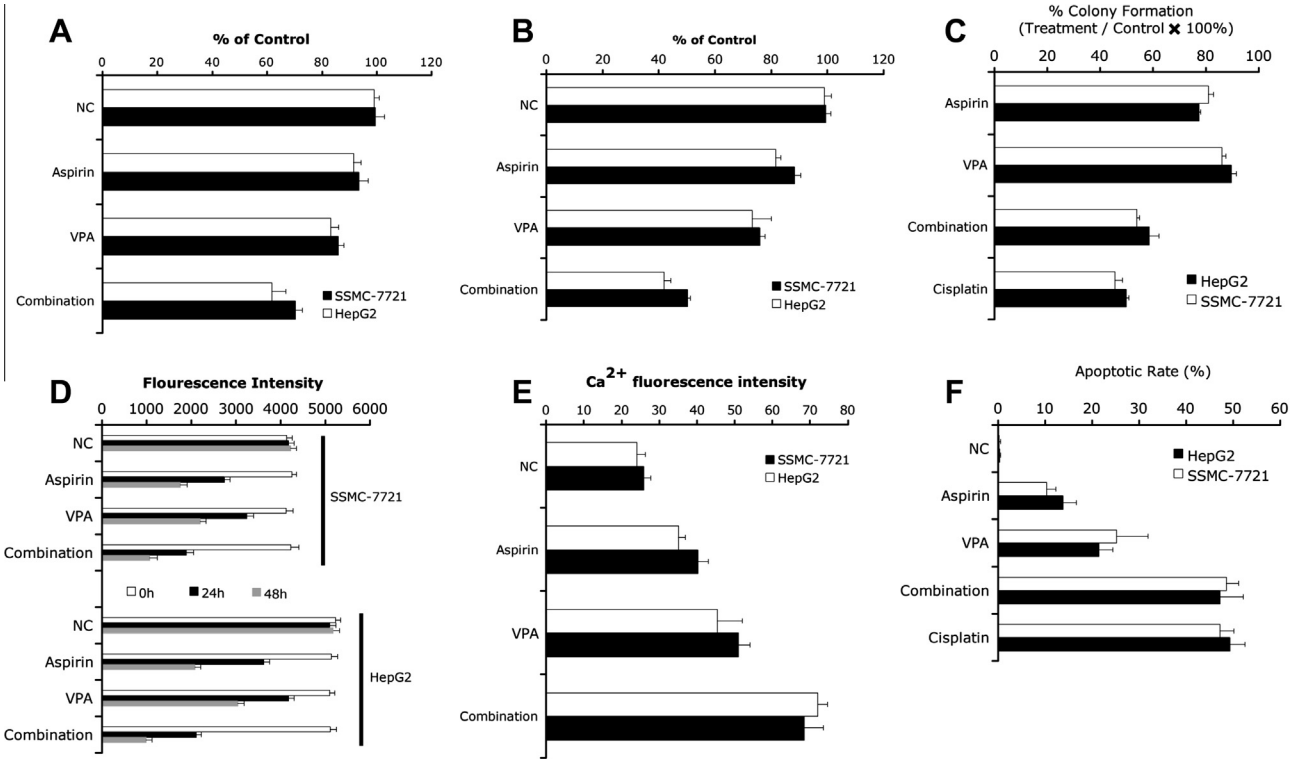

Fig. 2. Motility/invasion, colony formation and apoptosis analysis. (A) Motility of HepG2 and SMMC-7721 cells on drug separately used or combination (4 μmol/L aspirin and 2.5 mmol/L VPA). (B) Invasion of HepG2 and SMMC-7721 cells under drug separately used or combination. (C) Colony formation of HepG2 and SMMC-7721 cells decreased significantly in combination group, $^{*}P < 0.05$. (D) Mitochondrial membrane potential assay on HepG2 and SMMC-7721 cells with its combination, $^{*}P < 0.05$. (E) Of Alternation of [Ca^{2+}] concentration in different treatment, $^{*}P < 0.05$. (F) Apoptotic rate of HepG2 and SMMC-7721 cells on different treatment, $^{*}P < 0.05$. NC indicated as negative control.

decreased the number of the colonies (Fig. 2C) from HepG2 and SMMC-7721 cell lines.

As combination of aspirin plus VPA caused a significant reduction in cell proliferation, motility and invasion, the underlying mechanisms were investigated. Mitochondrial membrane potential and [Ca^{2+}] alteration have been suggested to cause apoptosis, in combination treatment, mitochondrial membrane potential decreased in the fluorescence intensity of CMXRos (Fig. 2D). Significant [Ca^{2+}] elevation was also observed in the combination group (Fig. 2E). Lastly, to confirm the induction of apoptosis by this combination, HepG2 and SMMC-7721 cells were treated with the drugs alone or in combination and examined by FACS analysis (Fig. 2F). At the concentrations tested, aspirin and VPA combination elicited significant apoptosis compared to drug used alone ($P < 0.05$).

3.3. Involvement of the extrinsic pathway in aspirin and VPA combination -mediated apoptosis

To further examine the processes of apoptosis induced by this combination, we analyzed cell extracts for mRNA (Fig. 3A) and protein (Fig. 3B) expression of biological markers of apoptosis. The combination drug treatment resulted in Bax and p15 increase, whereas Bcl-2, Survivin, Cyclin D1 and DNMT3A decrease. Studies shown that Hsp 70 may play an anti-apoptotic role by blocking the recruitment of procaspase-9 to the Apaf-1/dATP/cytochrome c apoptosome complex [17,18], therefore, HSP70 were also evaluated using RT-PCR (Fig. 3C) and Western blotting (Fig. 3D), HSP70 expression decreased significant in combination treatment. These results demonstrate that aspirin inhibited the proliferation of hepatocellular cells via an HSP70-independent pathway.

3.4. Combination inhibited in vivo hepatocellular cancer growth

In order to evaluate whether combination could inhibit tumor growth, the in *vivo* activity of combination on hepatocellular cancer cells was also evaluated. Subcutaneous inoculation of HepG2 (Fig. 4A and B) or SMMC-7721 (Fig. 4C and D) cells into nude mice resulted in a tumor formation at the site of injection in all mice. In these mouse xenograft models of hepatocellular cancer, tumor weight (Fig. 4A and C) and volume (Fig. 4B and D) in combination group was significantly decreased compared with single drug group ($P < 0.05$).

4. Discussion

In this study, the proliferative capacity of hepatocellular cancer cell line was significantly inhibited by aspirin and VPA combination. Meaning while, this combination does not have a side effect on normal hepatocellular cell proliferation *in vitro* at the therapeutic dose used in the cancer cell line treatment. The results of the present study also suggest that tumor motility/invasion could be attenuated treated by this combination. Further studies are need to be done to elucidate the mechanisms of motility/invasion suppression by aspirin and VPA combination, further studies in other cancer cell lines are also need. Moreover, this combination not only highly blocked the hepatocellular cancer cell lines' proliferation, but also apoptosis was induced. Lastly, *in vivo* studies, tumor growth in bearing mouse could be inhibited by the combination therapy.

Although aspirin was originally synthesized and widely used for a long period, it's possible mechanisms of anticancer activity are diverse and not well understood. The COX signaling pathway involved in critical growth and development process of cancers,

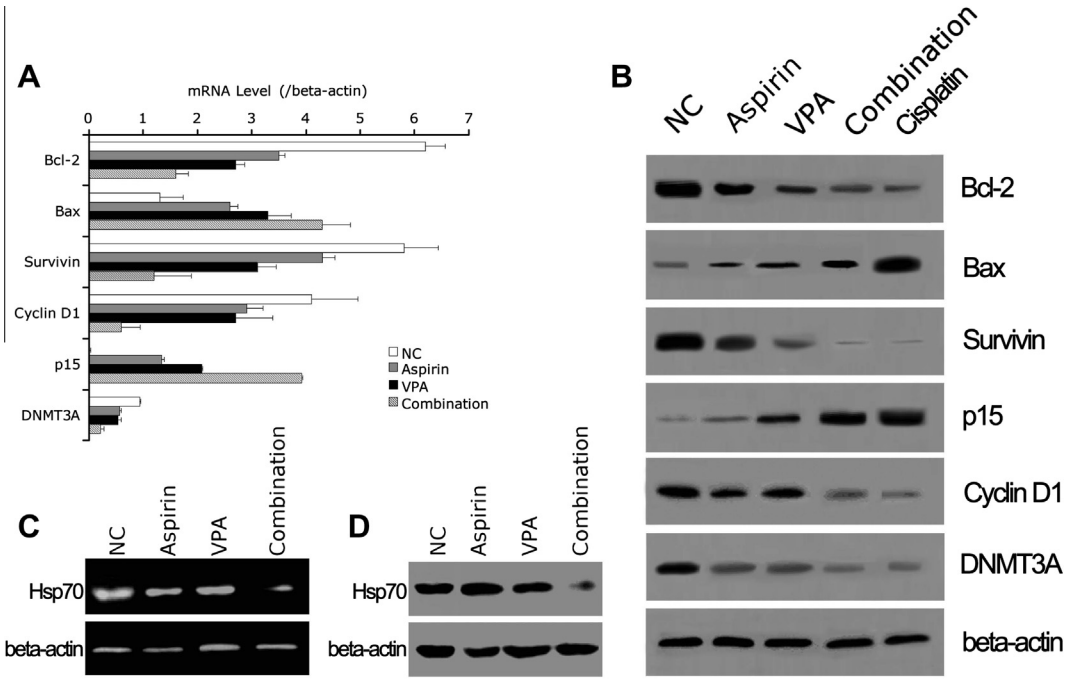

Fig. 3. Gene expression analysis after drug treatment. (A) Real-time PCR analysis on HepG2 cells' cycle and apoptosis related genes' expression level, Cyclin D1, DNMT3A and Bcl-2 decreased while p15 and Bax expression increased in combination (4 μmol/L aspirin and 2.5 mmol/L VPA). (B) Western blotting assay on HepG2 cells' cycle and apoptosis related genes' expression. (C) RT-PCR analysis on Hsp 70 expression. (D) Western blotting assay on Hsp 70 expression. NC indicated as negative control.

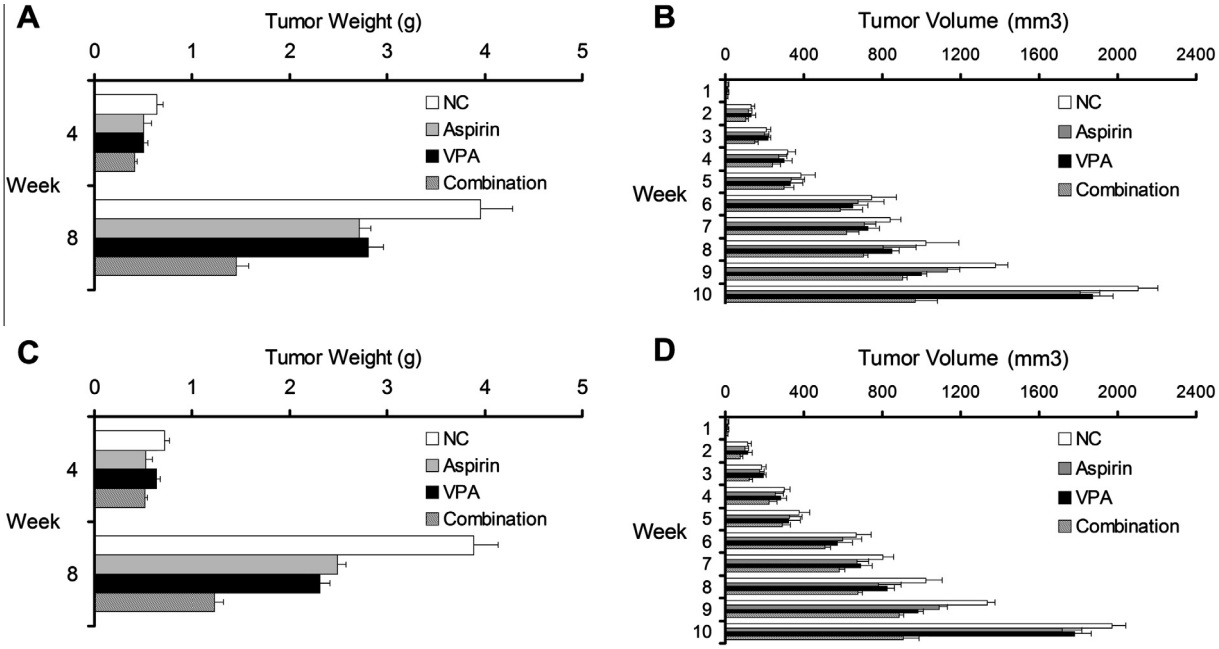

Fig. 4. Hepatocellular model therapy. (A) Tumor (HepG2 bearing mouse model) weight under different treatment conditions, $^{*}P < 0.05$. (B) Tumor (HepG2 bearing mouse model) volume of mouse model on different therapies, $^{*}P < 0.05$. (C) Tumor (SMMC-7721 bearing mouse model) weight under different treatment, $^{*}P < 0.05$. (D) Tumor (SMMC-7721 bearing mouse model) volume of mouse model for different therapies, $^{*}P < 0.05$. NC indicated as negative control.

including angiogenesis, apoptosis, cell proliferation and migration, thus, putative COX-dependent anticancer effects of aspirin are supported by the emerging clinical data from both the primary and secondary prevention setting [19]. However, novel studies showed that the anticancer properties of aspirin was associated with the suppression of antiapoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) and inhibitors of apoptosis related proteins (XIAP and Survivin) in cancer cells [20], and moreover, observation have shown that COX-2 inhibition reduces Hsp70 expression during papillary cells apoptosis [21],

Inhibiting HDAC activity modulates the epigenetic status of cells, resulting in an alteration of gene expression and cellular function, and HDACs are aberrantly expressed in different cancers. Application of VPA to the colon adenocarcinoma not only led to a significant suppression of vascular endothelial growth factor (VEGF) expression amd secretion [22]. Moreover, VPA was found

to modulate the MAP kinase pathway which plays a key role in the development of cancer [23]. VPA inhibited glycogen and RNA ribose turnover and disrupted glucose-derived cholesterol synthesis in mice which will deprive the tumor from cholesterol which is needed for tumor growth [24]. Additionally, Hsp70 expression decrease can be induced by VPA in anti-inflammatory [25] or neuroprotective effects [26].

Hsp 70, a client protein, includes kinases or transcription factors involved in signal transduction, in numerous tumor cells, is overexpressed and forms multi-chaperone complexes with client proteins that are involved in processes characteristic to malignant phenotypes, such as invasion, angiogenesis and metastasis [27]. These open the question of whether Hsp 70 play an important role in growth suppression and apoptosis induction in aspirin and VPA combination. From our the mechanism exploration on hepatocellular cancer cells apoptosis by aspirin and VPA combination treatment, we conclude that synergistic effect of drug combination may be through the decreasing Hsp 70 expression, then damaged the growth or cell cycle related proteins and apotosis related proteins to suppress hepatocelluar cancer cells, however, the detail of Hsp 70 in the synergistic effect still have a lot of work to do.

In summary, our present results have demonstrated that the combination of aspirin and VPA significantly enhanced the inhibition of hepatocelluar cancer cell proliferation and migration, and induced apoptosis and cell cycle arrest by targeting the Hsp 70 mediated signaling pathways of COX in hepatocelluar cancer cells. The combination of aspirin and VPA did not show significant inhibition of the growth of the normal hepatocelluar cells, moreover, aspirin and VPA combination can synergistically inhibit hepatocelluar tumor growth in vivo. All these findings suggest that the combination of aspirin and VPA may have the wide therapeutic and/or adjuvant therapeutic application in the treatment of hepatocelluar cancer in clinical.

Conflict of interest

The authors have no commercial or financial interest in the products or companies described in this article.

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