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# Effects of aspirin on proliferation, invasion and apoptosis of Hep-2 cells via the PTEN/AKT/NF-κB/survivin signaling pathway

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**Abstract.** Aspirin may exhibit antitumor activities, as it is able to inhibit cell proliferation. However, the ability of aspirin to inhibit cellular proliferation in Hep-2 cells and its underlying molecular mechanisms have been poorly determined. The aim of the present study was to investigate whether aspirin may induce cell apoptosis in the neoplastic cell line Hep-2. The effects of aspirin on the migratory and invasive abilities of Hep-2 cells were also investigated using Transwell assays. In the present study, it was demonstrated that aspirin induced apoptosis and inhibited proliferation, migration and invasion in Hep-2 cells. Aspirin also significantly decreased the expression of B-cell lymphoma 2 (Bcl-2) and caspase-3, and increased the expression of Bcl-2-associated X protein, suggesting that aspirin induced apoptosis through the intrinsic apoptotic pathway. Hep-2 cells treated with aspirin exhibited a significant upregulation of phosphatase and tensin homolog (PTEN) and decreased levels of phosphorylated protein kinase B (AKT). However, the total amount of AKT protein was not altered in response to aspirin treatment. Furthermore, the expression of nuclear factor (NF)-κB and survivin, which are the downstream targets of the PTEN/AKT signaling pathway, was inhibited. These results indicated that the molecular mechanism underlying the antitumor effects of aspirin may be associated with the inhibition of tumor invasion and induction of apoptosis by regulating the activity of the PTEN/AKT/NF-κB/survivin signaling pathway.

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## Introduction

Cancer constitutes a global burden and has become a serious health challenge (1,2). The occurrence of cancer is increasing due to an increasing aging population and prevalence of established risk factors (3-6). Although over the last few decades, novel therapeutic strategies have been developed, the prognosis and survival rate for patients with cancer is far from satisfactory. Therefore, investigating the molecular mechanisms underlying tumorigenesis has become urgent, particularly for the targeted treatment of human cancer. Investigation of the underlying molecular mechanisms is required to identify novel therapeutic targets for cancer.

Aspirin, which is one of the most common therapeutic drugs worldwide, was primarily introduced as an anti-inflammatory and analgesic agent (7). Nevertheless, clinical and epidemiological studies have demonstrated that aspirin may decrease the risk of several types of cancer, including colorectal, breast, prostate, lung and skin cancer (8-12). The anticancer effects of aspirin may be dependent on its ability to induce apoptosis or inhibit proliferation in cancer cells. To the best of our knowledge, there is no evidence to indicate whether aspirin is able to promote apoptosis and inhibit the proliferation and invasion of Hep-2 cells. The Hep-2 cell line is a mixed neoplastic type of cell type originally considered to derive from an epidermoid carcinoma of the larynx, but it has been demonstrated to be contaminated with cervical adenocarcinoma HeLa cells (13-15). Therefore, investigating the effect of aspirin on Hep-2 cells in this general model of cancer may be beneficial to prevent drug resistance in cancer therapy and develop novel tumor-targeted drugs.

The aim of the present study was to investigate the effects of aspirin on apoptosis, proliferation and invasion in Hep-2 cells. Furthermore, the expression of survivin, a key anti-apoptotic regulator of the phosphatase and tensin homolog (PTEN)/protein kinase B (AKT)/nuclear factor (NF)-κB signaling pathway, was examined. The results demonstrated that aspirin promoted apoptosis and inhibited proliferation and invasion of Hep-2 cells. Additionally, aspirin increased the B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax)/Bcl-2 ratio and activated caspase-3. These results indicate that the

proliferation and metastasis may be partly regulated by aspirin in Hep-2 cells. Collectively, these results not only contribute to an improved understanding of the molecular mechanisms of apoptosis underlying the anticancer effect of aspirin, but also suggest therapeutic targets for several types of cancer.

## Materials and methods

*Cell culture*. Hep-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 $\mu$g/ml penicillin/streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay*. The drug half-maximal inhibitory concentration ($IC_{50}$) values were determined by using MTT. In brief, $1x10^4$ of Hep-2 cells were plated in a 96-well plate and were treated with aspirin (10, 50, 100 and 200 $\mu$g/ml) or 10 $\mu$M bovine papillomavirus (BPV; both Beyotime Institute of Biotechnology) for different time points. Cells were then incubated with 10 $\mu$l 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 4 h. The purple formazan crystals that formed were dissolved in 100 $\mu$l dimethyl sulfoxide and added to the cells. Absorbance was determined at a wavelength of 490 nm using a multi-plate reader (Synergy 2, BioTek Instruments, Inc., Winooski, VT, USA) and drug half-maximal inhibitory concentration ($IC_{50}$) values were established.

*Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay*. Hep-2 cells ($1x10^4$) were washed three times with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, permeabilized in 0.1% Triton X-100 in sodium citrate buffer. Apoptotic cells were labeled using an In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's protocol at room temperature for 15 min and the nuclei were stained with 0.3 mmol/l DAPI at room temperature for 5 min. TUNEL-positive cells were observed in 6 randomly selected fields under the Olympus BX50 (Olympus Corporation, Tokyo, Japan) fluorescence microscope (magnification, x200). The apoptotic rate, which was defined as the ratio of apoptotic cells to total cells, was calculated and analyzed using Image-Pro Plus 6.0 software (Nikon Corporation, Tokyo, Japan).

*Flow cytometric analysis of apoptosis*. Hep-2 cells ($1x10^5$) were plated in a 6-well plate for 24 h and then treated with aspirin, aspirin plus 10 $\mu$M BPV or saline (control). Cells were subsequently incubated at room temperature with Annexin V-fluorescein isothiocyanate and propidium iodine (PI; BD Biosciences, Franklin Lakes, NJ, USA) for 15 min before being analyzed on the Accuri™ C6 flow cytometer (BD Biosciences) using FlowJo, version 7.6.1 (FlowJo LLC, Ashland, OR, USA).

*Transwell migration and invasion assay*. The migration and invasion assay was carried out using Transwell plates (EMD Millipore, Billerica, MA, USA). The filter surfaces (8 $\mu$m pores) of the Transwell plates were precoated with 25 mg Matrigel at 4˚C overnight. Culture medium (DMEM) supplemented with 10% FBS was placed in the lower chambers. Hep-2 cells ($1x10^4$) were placed in the upper chambers of the Transwell plates without FBS. Following incubation for 24 h, invaded cells were fixed with 4% paraformaldehyde and stained with Giemsa at room temperature for 5 min. Three replicates were used for each condition. Stained cells were counted in 15 random fields using light microscopy (magnification, x200; CKX41; Olympus Corporation, Tokyo, Japan). The results are presented as a ratio relative to the control group.

*Western blot analysis*. Total protein was extracted from Hep-2 cells using RIPA buffer (Beyotime Institute of Biotechnology). Total protein was quantified using bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) and 80 $\mu$g protein/lane was separated using SDS-PAGE (10% gels) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk dissolved in PBST for 2 h at room temperature and incubated with the following primary antibodies: Anti-Bcl-2 (15071; 1:1,000), anti-Bax (5023; 1:1,000), anti-caspase-3 (9662; 1:1,000), anti-PTEN (9559; 1:1,000), anti-AKT (9272; 1:1,000), anti-phospho (p)-AKT (4060; 1:1,000), anti-NF-κB (8242; 1:1,000), anti-survivin (2808; 1:1,000; all Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-actin (612657; 1:1,000; BD Biosciences, Franklin Lakes, NJ, USA) diluted in PBS and incubated at 4˚C overnight. Membranes were then washed with Tris-buffered saline with Tween-20 (TBST) and incubated with goat anti-rabbit IgG secondary antibody (Alexa Fluor-conjugated; A32730; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using Odyssey software version 1.2 (LI-COR Biosciences). GAPDH was used as an internal control.

*Statistical analysis*. Data were analyzed using GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean. Results were analyzed using an unpaired two-tailed Student's t-test and one-way analysis of variance followed by Bonferroni's correction for multiple comparison tests. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Aspirin significantly decreases viability in a dose- and time-dependent manner in Hep-2 cells*. In order to examine the effect of aspirin on the viability of Hep-2 cells, MTT assays were employed. Hep-2 cells were treated with 0, 10, 50, 100 and 200 $\mu$g/ml aspirin for 48 h. The $IC_{50}$ value of aspirin was identified to be 91.2 $\mu$g/ml (Fig. 1A); therefore, 100 $\mu$g/ml was selected as an optimal concentration for subsequent experiments. Hep-2 cells were treated with 100 $\mu$g/ml aspirin for 12, 24 and 48 h (Fig. 1B). The results demonstrated that

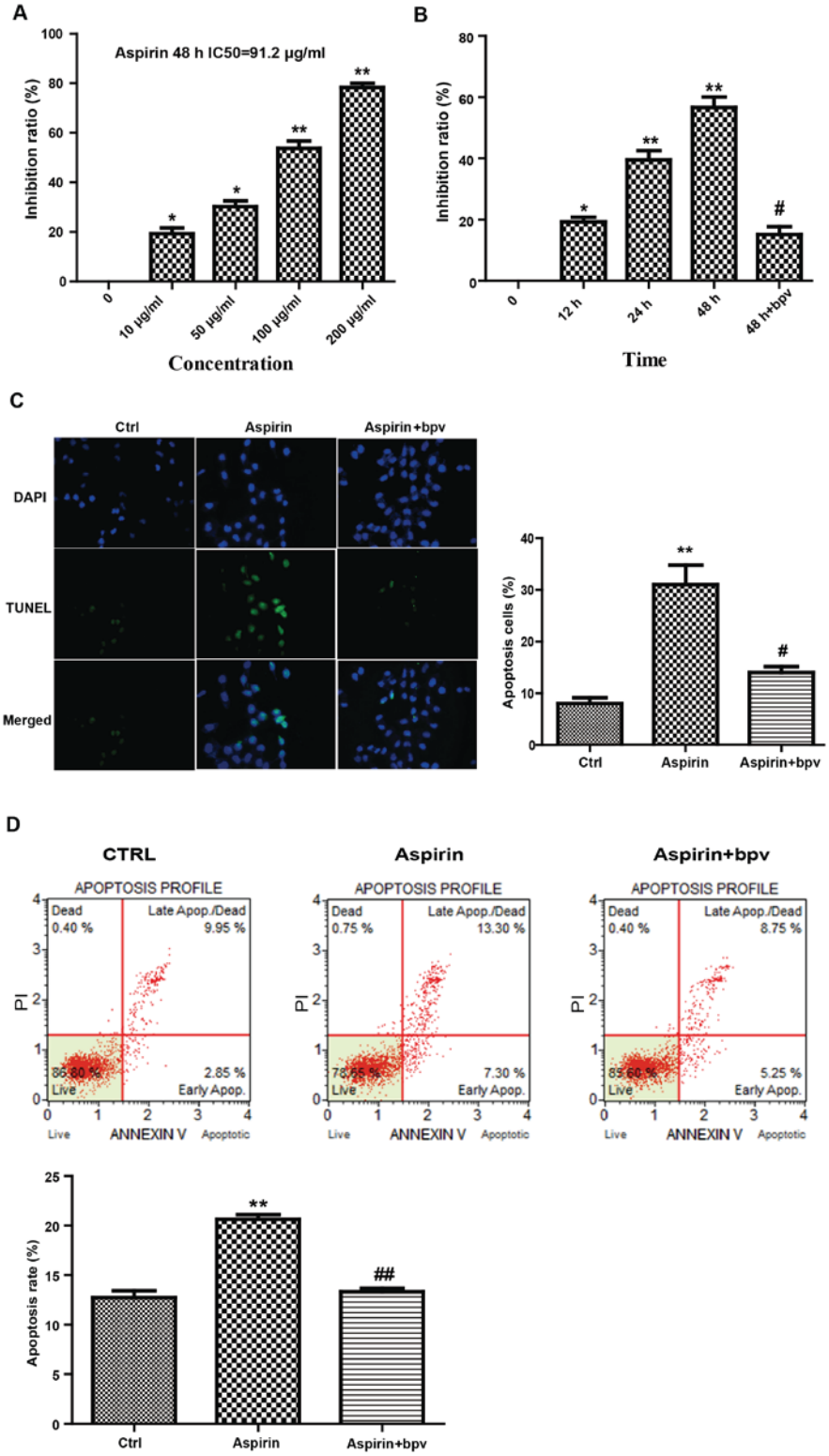


Figure 1. Aspirin inhibits viability and induces apoptosis in Hep-2 cells. (A) Cells were treated with 0, 10, 50, 100 and 200 $\mu$g/ml aspirin for 48 h, and cell viability was assessed using an MTT assay. (B) Cells were treated with 100 $\mu$g/ml aspirin for 12, 24 and 48 h, and cell viability was assessed using an MTT assay. (C) Aspirin promotes cell apoptosis in Hep-2 cells as demonstrated using a TUNEL assay. (D) Aspirin promotes apoptosis in Hep-2 cells as demonstrated using flow cytometric analysis of apoptosis. n=6; $^{*}$P<0.05 vs. control; $^{**}$P<0.01 vs. control; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. aspirin. MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; BPV, bovine papillomavirus; apop., apoptosis; Ctrl, control.

aspirin significantly decreased the viability of Hep-2 cells in a dose- and time-dependent manner (Fig. 1A and B; P<0.05). However, further *in vivo* and *in vitro* experiments are required to confirm these results.

Since aspirin is a potential agonist of PTEN (16), the present study investigated whether aspirin may decrease cell viability via regulating the PTEN signaling pathway. Therefore, cells were treated with 100 $\mu$g/ml aspirin and

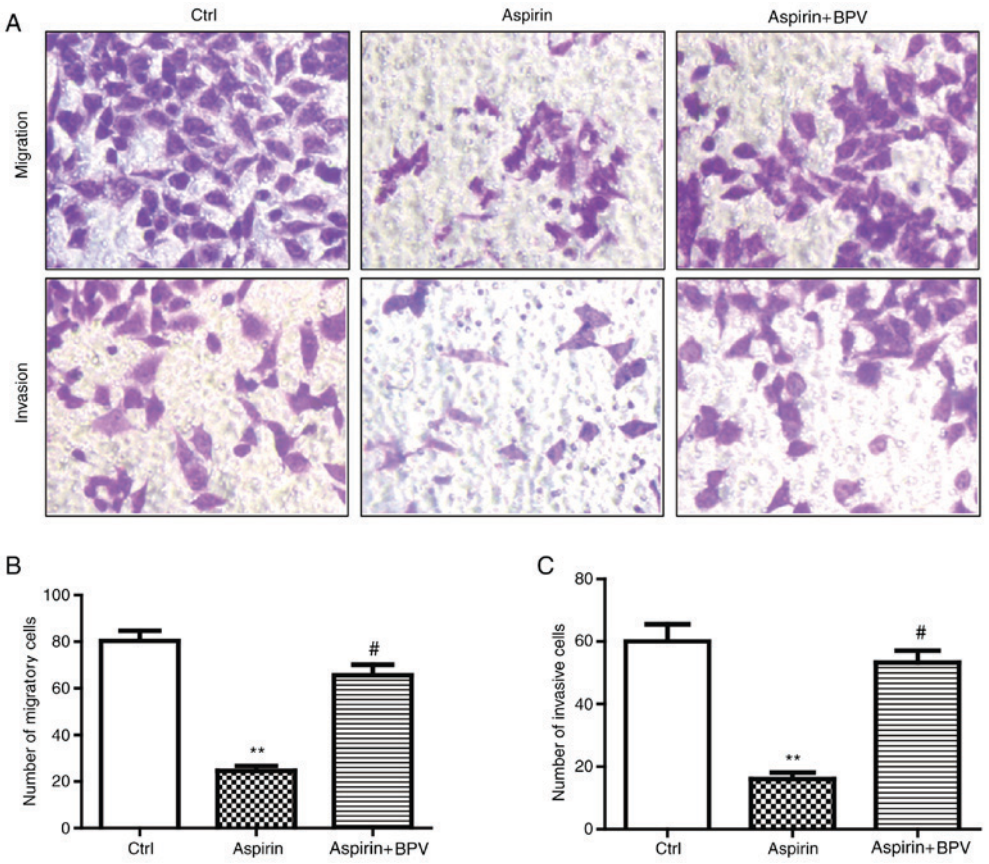


Figure 2. Effects of aspirin on the migration and invasion of Hep-2 cells. (A) Representative images of migrating and invading cells in control, aspirin and aspirin plus BPV groups (magnification, x200). (B) Average number of migrated cells in control, aspirin and aspirin plus BPV groups. (C) Average number of invading cells in control, aspirin and aspirin plus BPV groups. Three independent experiments were performed. n=3; $^{**}$P<0.01 vs. Ctrl; $^{\#}$P<0.05 vs. aspirin. BPV, bovine papillomavirus; Ctrl, control.

10 $\mu$M BPV, an inhibitor of PTEN, for 48 h, and cell viability was assessed using an MTT assay. As presented in Fig. 1B, combined treatment with aspirin and BPV significantly decreased the inhibition in Hep-2 cells, suggesting that the PTEN signaling pathway may be a molecular mechanism underlying aspirin-mediated cellular changes in Hep-2 cells.

*Aspirin promotes apoptosis in Hep-2 cells*. To evaluate the effect of aspirin on apoptosis, apoptotic cells were stained using a TUNEL assay. The results demonstrated that treatment with aspirin significantly increased the numbers of apoptotic cells in Hep-2 cells (Fig. 1C) compared with in the control group (P<0.05). Additionally, BPV reversed the pro-apoptotic effects of aspirin in Hep-2 cells (Fig. 1C).

The apoptosis results were confirmed by flow cytometric analysis. As presented in Fig. 1D, aspirin induced an increase in apoptosis compared with that in the control group. Additionally, combined treatment with aspirin and BPV decreased the number of apoptotic cells compared with aspirin treatment (P<0.05).

*Aspirin inhibits the migratory and invasive abilities of Hep-2 cells*. Transwell assays were employed to investigate the ability of Hep-2 cells to migrate to and invade the extracellular matrix. The results demonstrated that the migration rate of Hep-2 cells decreased by >60% compared with that in the control group (Fig. 2A and B). However, combined treatment with aspirin and BPV did not affect the migratory ability of Hep-2 cells compared with those in the control group (Fig. 2B).

The effect of aspirin on cell invasion was investigated. As presented in Fig. 2C, the number of invasive cells was decreased by ~70% in response to treatment with aspirin in Hep-2 cells compared with those in the control group. Taken together, these results indicated that aspirin significantly inhibited cell invasion and migration *in vitro* and these effects were reversed when PTEN expression was downregulated.

*Involvement of the PTEN/AKT/NF-κB/survivin pathway in aspirin-induced apoptosis in Hep-2 cells*. To determine the molecular mechanism underlying the pro-apoptotic effects of aspirin, Hep-2 cells were treated with aspirin alone or with aspirin and BPV, and the expression levels of proteins involved in the PTEN/AKT/NF-κB/survivin signaling pathway were determined using western blot analysis. As presented in Fig. 3A, treatment with aspirin significantly increased the expression level of PTEN compared with that in the control group. Additionally, aspirin significantly decreased the relative expression level of p-AKT without affecting the expression levels of total AKT in Hep-2 cells (Fig. 3B and C). Furthermore, as presented in Fig. 3D and E, the expression levels of NF-κB and survivin were significantly decreased in the aspirin group compared with that in the control group (P<0.05). However, combined treatment with aspirin and BPV did not affect the expression levels of these proteins compared with the control

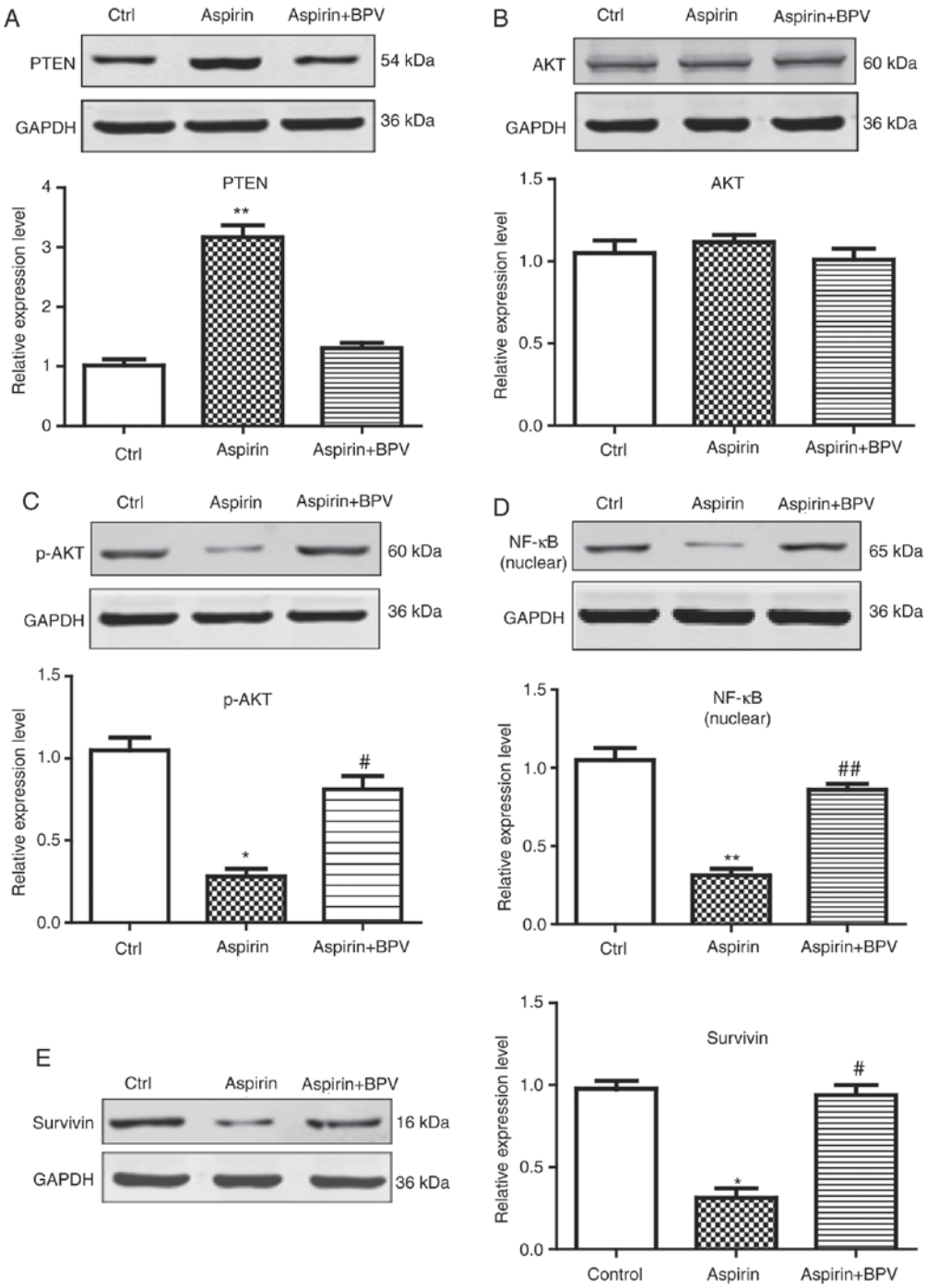


Figure 3. Effects of aspirin on the PTEN/AKT/NF-κB/survivin signaling pathway in Hep-2 cells. (A) Aspirin upregulated the expression of PTEN in Hep-2 cells. (B) Aspirin did not affect the expression of total AKT. (C) Aspirin decreased the expression of p-AKT. (D) Aspirin decreased the expression of p-NF-κB in Hep-2 cells. (E) Aspirin decreased the expression of survivin. n=6; $^{*}$P<0.05 or $^{**}$P<0.01 vs. Ctrl; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. aspirin. BPV, bovine papillomavirus; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; p, phospho; NF, nuclear factor; Ctrl, control.

group (P>0.05). These results indicated that aspirin decreased the protein expression of NF-κB and survivin by inactivating the PTEN/AKT signaling pathway in Hep-2 cells.

The effects of aspirin on the expression of downstream apoptotic proteins of the PTEN/AKT/NF-κB/survivin signaling pathway were investigated in Hep-2 cells. The Bcl-2 family proteins include the pro-apoptotic Bax and anti-apoptotic Bcl2 proteins (17). Hep-2 cells were incubated with aspirin or with aspirin and BPV for 24 h. The results demonstrated that Hep-2 cells treated with aspirin alone exhibited increased expression levels of Bax and decreased expression levels of Bcl-2 compared with those in the control group (Fig. 4A). Additionally, aspirin treatment induced a >3-fold increase in the Bax/Bcl-2 ratio compared with that in control group, indicating that aspirin induces apoptosis in Hep-2 cells.

Caspase-3 is a major caspase and its activation leads to cell death (18). Therefore, the expression levels of caspase-3 in response to treatment with aspirin were evaluated using western blot analysis. The results revealed that the expression

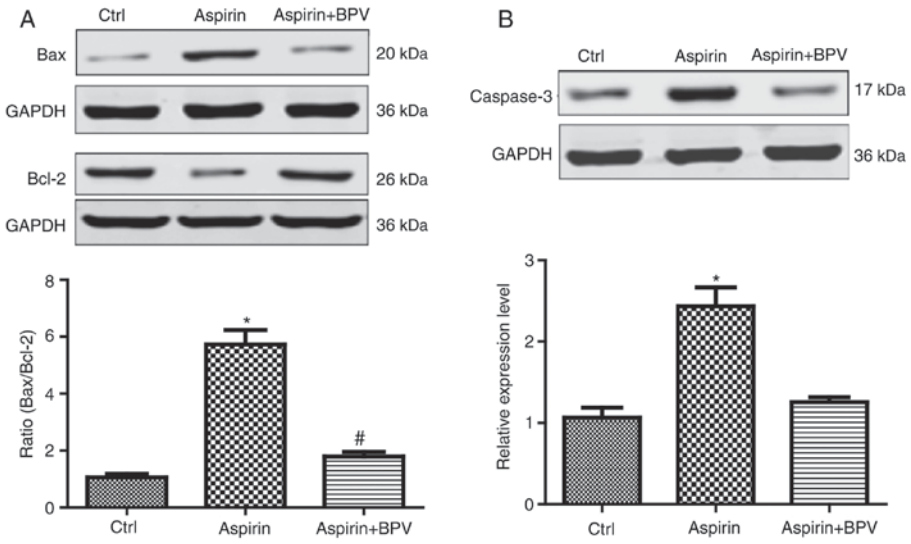


Figure 4. Effects of aspirin on the apoptosis-associated proteins in Hep-2 cells using western blot analysis. (A) Aspirin increases the Bax/Bcl-2 ratio in Hep-2 cells. (B) Aspirin increases the expression of caspase-3 in Hep-2 cells. n=6; *P<0.05 vs. Ctrl; #P<0.01 vs. aspirin. BPV, bovine papillomavirus; Ctrl, control; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

of caspase-3 was significantly upregulated in response to treatment with aspirin in Hep-2 cells (Fig. 4B). However, treatment with BPV attenuated the pro-apoptotic effects of aspirin in Hep-2 cells (Fig. 4B). Taken together, these results suggest that aspirin markedly induced apoptosis in Hep-2 cells by inhibiting the PTEN/AKT/NF-κB/survivin signaling pathway.

## Discussion

Malignant tumors are a major health concern. Drug resistance and relapse are the main reasons for poor prognosis and low survival rate. Therefore, there is an urgent requirement to elucidate the underlying molecular mechanisms of drug resistance, cell proliferation and metastasis in order to identify novel therapeutic approaches and improve the prognosis for patients with cancer. In the present study, it was demonstrated that aspirin induced apoptosis and suppressed the invasive and migratory abilities of Hep-2 cells. Additionally, aspirin mediated cellular proliferation, invasion and apoptosis via regulating the expression of the PTEN/AKT/NF-κB/survivin signaling pathway *in vitro*. These results provide insights into the molecular mechanisms of aspirin to regulate the proliferation, invasion and apoptosis in Hep-2 cells, but also suggest novel therapeutic targets for the treatment of various types of cancer.

Aspirin, also known as acetylsalicylic acid, is a non-steroidal anti-inflammatory drug, which is non-toxic to humans (19). Previous clinical and epidemiological studies have demonstrated that long-term treatment with aspirin may decrease the risk of colorectal, breast, prostate, lung and skin cancer (8,10,12). However, whether aspirin may promote cellular apoptosis and inhibit proliferation and invasion remains unclear. The aim of the present study was to investigate the effects of aspirin on cell apoptosis, proliferation and invasion in Hep-2 cells. The results demonstrated that the molecular mechanism by which aspirin induced apoptosis and inhibited proliferation and invasion may be associated with changes in the expression of Bcl-2, Bax and caspase-3. Additionally, the PTEN/AKT/NF-κB/survivin signaling pathway may have a function in the molecular mechanism underlying aspirin-induced cellular changes.

PTEN is a key molecule involved in glucose metabolism, cellular differentiation, proliferation, apoptosis and migration and in inflammatory response by targeting AKT and NF-κB. The PTEN/AKT signaling pathway leads to deregulation of the cell cycle and to apoptosis, and induces the differentiation of normal cells into tumor cells (20-22). In the present study, PTEN was selected as a potential target for aspirin to investigate whether aspirin may be involved in the PTEN/AKT signaling pathway in Hep-2 cells. The results demonstrated that aspirin upregulated the expression of PTEN and inhibited p-AKT, whereas total AKT expression was not significantly altered. Additionally, aspirin inhibited the expression of proteins downstream of the PTEN/AKT signaling pathway, including NF-κB and survivin. Additionally, aspirin increased the expression of Bcl-2, and decreased the expression of Bax and caspase-3 in Hep-2 cells. Collectively, these results demonstrate that aspirin serves a pro-apoptotic function by activating the PTEN/AKT/NF-κB/survivin signaling pathway in Hep-2 cells. Aspirin-mediated cellular changes were partially inhibited in response to a PTEN inhibitor, indicating that the PTEN/AKT/NF-κB/survivin signaling pathway may be an important target for the development of novel therapeutic strategies for cancer.

The results of the present study identified that aspirin was able to suppress proliferation, migration and invasion, and promote apoptosis of Hep-2 cells via the PTEN/AKT/NF-κB/survivin signaling pathway. Thus, aspirin has a critical effect in modulating cell proliferation, migration and apoptosis, and may serve as a potential therapeutic target for patients with cancer.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author's contributions

MJ was responsible for the design and writing of the article, and performance of experiments. CL performed the TUNEL assay and flow cytometric analysis of apoptosis. QZ and SX performed western blotting, and Transwell migration and invasion assays. XK contributed to data analysis. JW constructed diagrams and was responsible for study conception and revision of the article. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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