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ORIGINAL ARTICLE

Effects of aspirin on hepatocellular carcinoma and its potential molecular mechanism

Zhilu Yuan[1], Jingfei Zhao[1], Zhengtang Wang[1], Guoyan Ren[2], Zhongbao Zhang[1], Genshun Ma[3]

[1]Department of General Surgery, the third People's Hospital of Liaocheng, Liaocheng 252000, China; [2]Department of Surgery, the third People's Hospital of Liaocheng, Liaocheng 252000, China; [3]Department of General Surgery, Liaocheng People's Hospital, Liaocheng 252000, China.

Summary

Purpose: *To explore the effects of aspirin (ASP) on the proliferation and apoptosis of HepG2 hepatocellular carcinoma (HCC) cells via the Wnt/β-catenin signaling pathway.*

Methods: *Human HCC cells were cultured and treated with ASP at different concentrations. Cell proliferation was determined with cell counting kit-8 (CCK-8) and colony formation, and the rate of apoptosis was measured by flow cytometry. Western blotting (WB) and quantitative polymerase chain reaction (qRT-PCR) assays were used to assess the changes in the expression levels of related proteins.*

Results: *ASP showed a time-and concentration-depented inhibitory effect on HepG2 cell proliferation. The number of colonies formed in ASP-treated HCC cells was significantly lower than in control cells. For HCC cells treated with ASP, the apoptosis rate enhanced with the increase of ASP concentration. The expression levels of TCF4 and LEF1, key molecules of the Wnt/β-catenin signaling pathway, were lowered in HCC cells treated with 4 mM ASP, and the nuclear translocation of β-catenin was weakened. The β-catenin activator exerted a negative influence on the anticancer effect of ASP.*

Conclusions: *ASP inhibits the proliferation and promotes the apoptosis of HCC cells through the Wnt/β-catenin signaling pathway.*

Key words: *aspirin, Wnt/β-catenin signaling pathway, hepatocellular carcinoma, proliferation, apoptosis*

Introduction

As a health problem mainly detected in cirrhotic liver worldwide, hepatocellular carcinoma (HCC) is usually induced by genetic factors, alcoholic injury and chronic infection like infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) [1]. It is the fifth most common cancer and the second largest contributor to cancer-related death, ending the lives of approximately 745,000 patients every year [2,3]. Due to the low early detection rate and high recurrence and metastasis rates, the overall outcome of HCC treatment tends to be unfavorable despite recent advances in treatment strategies. Chemotherapy is commonly adopted for end-stage HCC at present. However, a large number of anticancer drugs show strong cytotoxicity and tend to induce drug resistance. Adefovir dipivoxil, entecavir, and their peers have been currently used in clinical trials of HCC, but the incidence rate of HCC has not decreased yet and even demonstrated an upward trend [4,5].

Acetylsalicylic acid (ASA), a non-steroidal anti-inflammatory drug, is equipped with various pharmacological merits such as anti-inflammatory, antipyretic and analgesic. Research has shown that long-term administration of ASA can help prevent colorectal cancer and other tissue malignant tumors [6]. It is indicated in several epidemiological studies and randomized controlled trials conducted

Corresponding author: Genshun Ma, MM. Department of General Surgery, Liaocheng People's Hospital, 67 Dongchang Rd, Liaocheng, Shandong, 252000, China.
Tel: +86 015095079930, Email: 12837113@qq.com
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in different populations that aspirin (ASP) is able to prevent the occurrence of adenoma, reduce the incidence and mortality rates of colorectal cancer, and improve the survival rate of colorectal cancer (CRC) patients [7-9]. It is considered that the anti-tumor effect of ASP is attributed to its ability to inhibit cell proliferation and induce apoptosis.

Recent well-founded data have supported that ASP can reduce the morbidity and mortality rates of other cancers and metastases [10,11]. These shreds of evidence strongly and unanimously support the anti-tumor effect of ASP, while the underlying mechanism is still little known. A large number of experimental data have manifested the role of ASP in regulating Wnt/β-catenin signal. Based on the role of Wnt/β-catenin signal in carcinogenesis and the potential effects of ASP on various cancers, ASP-mediated effects *via* this way have shown a greater correlation with such a way.

Studies have indicated that the overexpression of β-catenin in HCC may activate the signaling pathway of downstream signals, thus regulating the bioprocesses including cell proliferation, apoptosis and invasion. There is still no report about the effect of ASP on the Wnt/β-catenin signaling pathway in HCC. Therefore, in this study, the effects of ASP on the proliferation and apoptosis of HepG2 cells and the expression of the Wnt/b-catenin signaling pathway protein in HCC were analyzed, and the possible regulatory effect of this interaction on HCC is discussed in depth, so as to optimize the application of ASP in clinical prevention and treatment.

Methods

Cell culture

Human HCC lines HepG2 and HCCLM3 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences, Shanghai, China. HepG2 cells and HCCLM3 cells were cultured in Dulbecco modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified atmosphere at 37°C. The culture medium was replaced every other day, and subculture was conducted when the cell confluence reached 80-90%.

Quantitative reverse transcription - polymerase chain reaction (qRT-PCR)

Total RNAs were isolated using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and purified using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to its instructions. They were quantified by measuring absorbance at 260 nm and 280 nm wavelengths, and complementary deoxyribonucleic acid (cDNA) was synthesized from them using a PrimeScript RT kit (TaKaRa, Tokyo, Japan). The polymerase chain reaction (PCR) was conducted with the specific primers shown in Table 1.

Cell proliferation assay

Referring to the manufacturer's instructions, cell proliferation was detected using a cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HepG2 cells (1×10^4 cells/well) were cultured in a 96-well plate with 100 μL DMEM containing 10% FBS. CCK-8 (10 μL/well) was added at 12 h, 24 h, 48 h and 72 h and then incubated at 37°C for 3 h. The

Table 1. Primer sequences

Gene	*Forward primer sequence*	*Reverse primer sequence*
TCF4	5CF4encevrimer Sequencevi	5CF4encevrimer Sequence
LEF1	5'-CTGGAGAATACAGAGC- 3'	5'- CCTGGCTCCTCACTTGGC-3'
β-actin	5'- AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

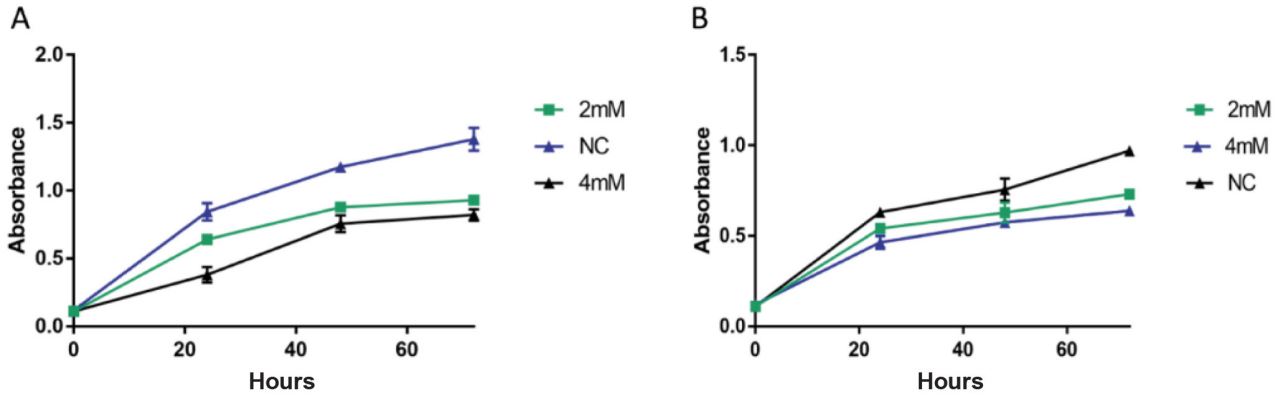

Figure 1. Cell proliferation assay: ASP shows a time- **(A)** and concentration-depented **(B)** inhibitory effect on HepG2 cell proliferation ($p<0.05$).

absorbance was detected at the 450 nm wavelength with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). All assays were repeated at least 3 times.

Flow cytometry assay for apoptosis

Cells were collected and seeded into a 6-well plates. Then they were digested by EDTA-free trypsin (Beyotime Biotechnology, Shanghai, China) and stained with Annexin V-FITC and propidium iodide (PI) (Shanghai Best-Bio Science, Shanghai, China). The cells were incubated for 15 min at room temperature in the dark. The cycle and apoptosis of cells in each group were detected by a EPICS XL-MCL flow cytometer (Beckman Coulter, Brea, CA, USA) with the excitation wavelength of 488 nm and the emission wavelength of 530 nm. A flow cytometer (FACScan, BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software (BD Biosciences, San Jose, CA, USA) was used to analyze these cells: living cells, dead cells, early apoptotic cells and late apoptotic cells were distinguished. In each assay, the percentages of early and late apoptotic cells were compared with those in control group respectively. The assay was repeated three times.

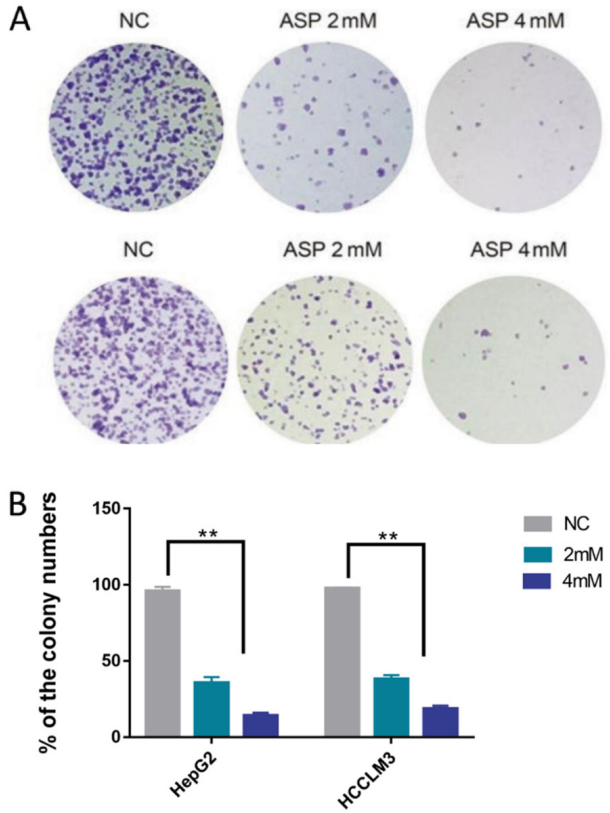

Figure 2. A: Colony formation experiment. **B:** The number of colonies formed in ASP-treated HCC cells is significantly lower than that in control cells (**p<0.01).

Western blotting (WB) analysis

Proteins were extracted with the radio immunoprecipitation assay (RIPA) buffer, separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (PVDF). After that, the membranes were sealed with 5% skim milk at 37°C for 2 h and incubated with primary antibodies at 4°C overnight. Membranes and the secondary antibodies conjugated with horseradish peroxidase were then incubated at 37°C for 1 h. Referring to the manufacturer's instructions, the bands were observed with an enhanced chemiluminescence detection kit.

Statistics

All statistical analyses were conducted using the SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA). All resulting data were expressed as mean ± standard deviation (SD) and analyzed by the two-tailed t-test and chi-square test. Comparison between multiple groups was done using one-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P<0.05 indicated that the difference was statistically significant.

Results

ASP inhibited the proliferation of HCC cells

To study the regulation of ASP on the proliferation of HCC line, HepG2 cells were selected for *in vitro* experiment. The above cell line was treated with ASP at different concentrations of 0, 2 and 4 mM for 24 h, 48 h and 72 h separately, and the cell activity was detected by CCK-8. Figure 1 shows that ASP showed a time- and concentration-depented inhibitory effect on HepG2 cell proliferation (p<0.05).

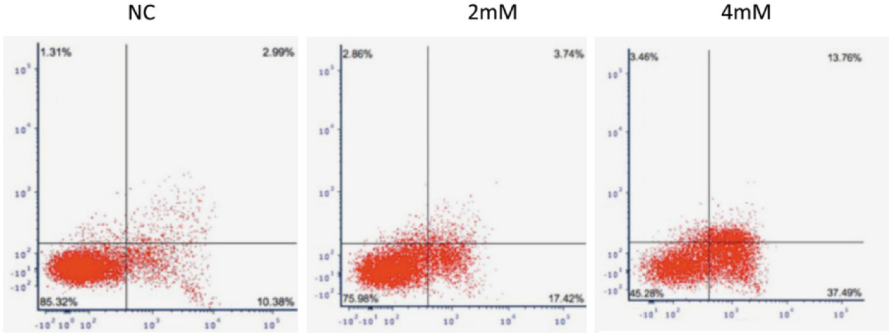

Figure 3. Apoptosis rate measured *via* flow cytometry assay: The apoptosis rate is 11.21 ± 1.22%, 21.3 ± 1.64% and 51.3 ± 12.64% in control group, 2 mM ASP group and 4 mM ASP group, respectively.

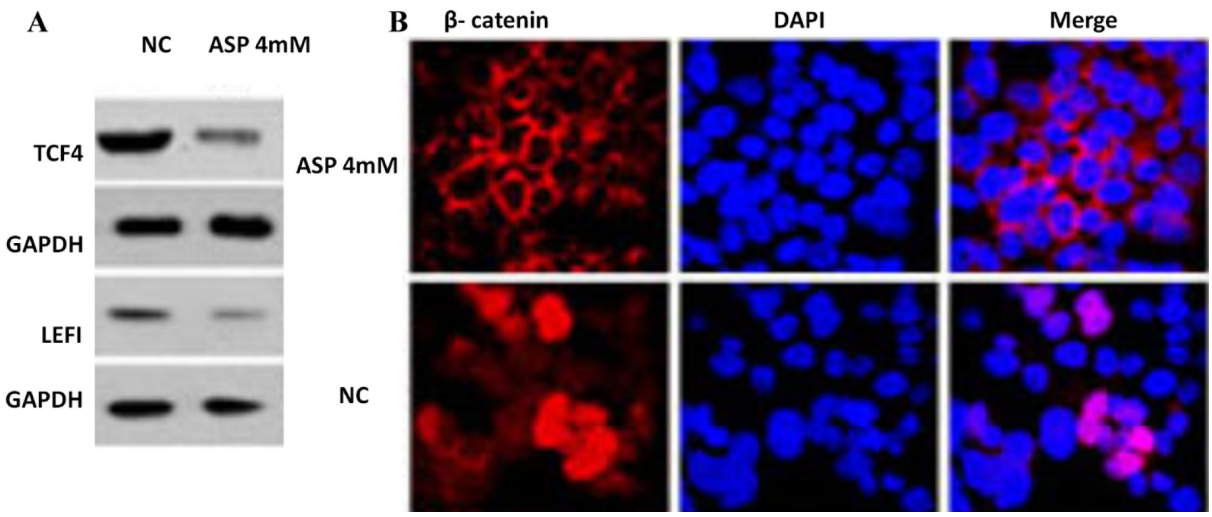

Figure 4. Effect of ASP on the Wnt/β-catenin signaling pathway. **A:** Results of WB show that the expression levels of TCF4 and LEF1 (the key molecules of the Wnt/β-catenin signaling pathway) are lowered in HCC cells treated with 4 mM ASP. **B:** The nuclear translocation of β-catenin is weakened after the treatment with ASP.

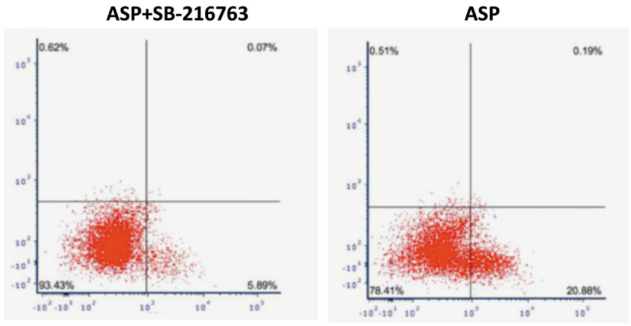

Figure 5. Apoptosis detected *via* flow cytometry: ASP combined with SB-216763 weakens the apoptosis of HCC cells.

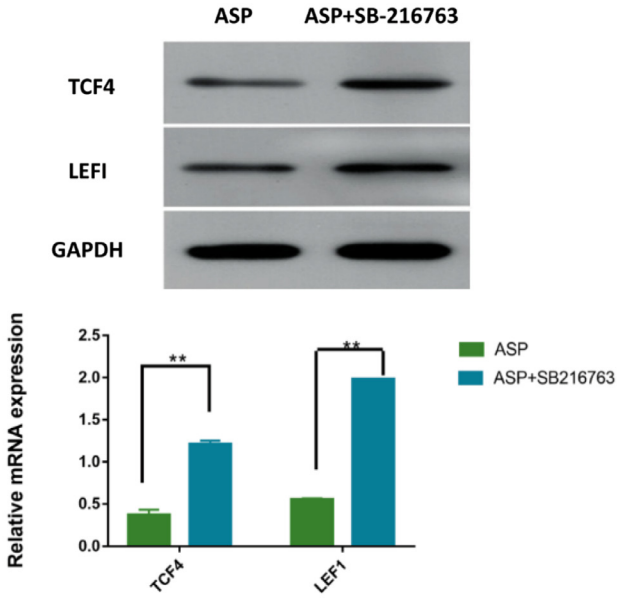

Figure 6. "Rescue" experiment: The expression of related molecules in the Wnt/β-catenin signaling pathway is enhanced after HCC cells are treated with ASP combined with SB-216763 (**p<0.01).

The viability of HepG2 cells was determined by colony formation assay, and the result indicated that the number of colonies formed in ASP-treated HCC cells was significantly lower than that in control cells ($p<0.05$) (Figure 2).

ASP promoted the apoptosis of HCC cells

HCC cells were treated with ASP at different concentrations, and then the apoptosis was detected. The results showed that in HCC cells treated with ASP, the apoptosis rate enhanced with the increase of ASP concentration. The apoptosis rate in the control group, 2 mM ASP group and 4 mM ASP group was 11.21±1.22%, 21.3±1.64% and 51.3±12.64%, respectively ($p<0.01$) (Figure 3).

ASP affected HCC cells via the Wnt/β-catenin signaling pathway

To further understand the molecular mechanism of ASP in affecting the proliferation and apoptosis of HepG2 cells, the expression of the Wnt/β-catenin signaling pathway related proteins was detected 48 h after HCC cells were treated with 4 mM ASP. The results showed that the expression levels of TCF4 and LEF1, the key molecules of the Wnt/β-catenin signaling pathway, were lowered in HCC cells treated with 4 mM ASP. In addition, the nuclear translocation of β-catenin was weakened after the treatment of ASP proved by the fluoroimmunoassay (Figure 4).

The β-catenin activator exerted a negative influence on the anticancer effect of ASP

The Wnt/β-catenin signaling pathway was activated by β-catenin activator SB-216763 to clarify the

effect of ASP on it. It was shown in the results that the apoptosis of HCC cells was weakened after the treatment with ASP and SB-216763 (Figure 5), and the expression of related molecules in the Wnt/β-catenin signaling pathway was enhanced (Figure 6).

Discussion

The genome-wide analysis on somatic mutation spectrum of various cancers carried out with the Cancer Genome Atlas Network brings the key role played by Wnt signaling in several kinds of tumors in light [12,13]. Furthermore, considerable evidence has shown that ASP may significantly produce chemoprophylaxis in other cancers related to the gastrointestinal tract (stomach and esophagus) [14]. Previous studies have pointed out that the potential interaction between ASP and Wnt signaling may have a certain relationship with the onset and development of various malignant tumors, and overactivation of Wnt signaling tends to be common in tumorigenesis. In addition, as mentioned above, developmental biology research on stem cells has supported that the PGE2/Wnt interaction plays a rather common part in different tissues. Moreover, the specific survival benefits equipped by ASP for tumor patients with PIK3CA mutation may make it more suitable for malignant tumors activating the Wnt signaling. In this study, ASP was found for the first time to inhibit the proliferation of HCC cells and stimulate their apoptosis, which stands as a support and supplement to previous studies.

In addition to its application as an adjuvant, ASP is also recommended to be used in combination with new chemotherapeutic drugs and biological agents being developed, which can perform special activities in regulating Wnt signaling. ASP and other non-steroidal anti-inflammatory drugs have successfully induced apoptosis of adenoma cells and human colon tumor cells *in vitro* that previously showed drug resistance by chemically sensitizing recombinant TNF-related apoptosis-inducing ligand (TRAIL) [15]. This sensitization requires the activation of Wnt signaling, further proving the dependence of ASP on the Wnt signaling pathway.

In this study, ASP was found to inhibit the proliferation of HepG2 cells in a dose-dependent manner and reached about 50% inhibition of cell viability at a dose of 600μM, with conclusions drawn from the determination of cell viability and colony formation. HCC exhibits distinct tumor heterogeneity, and many pathways are involved in maintaining the stem cell nature of HCC cells, including RAF/MEK/ERK, PI3K/AKT/mTOR and Wnt/β-catenin [16,17]. In this study, the Wnt/β-catenin signaling pathway was observed to be highly activated only when exposed to the carrier, which is consistent with the findings of previous studies. The constitutive activation of Wnt/β-catenin signaling pathway with abnormal accumulation of β-catenin is found in HepG2 cells [18]. As an important component of typical Wnt signaling, β-catenin can help activate the gene transcription [19]. It has been proved that β-catenin can be used as a co-transcription factor of its own promoter with the Wnt ligand [20]. Therefore, the decrease of β-catenin protein observed in cells in the study may be caused by positive feedback regulation. Various growth factors, TGF-β for example, can activate the β-catenin signaling pathway by autocrine Wnt ligands [21].

With the extensive interaction between ASP and Wnt/β-catenin being explored, whether ASP can be used in coordination with newly developed drugs that can inhibit the Wnt/β-catenin activity has thus drawn widespread attention. The anti-inflammatory effect allows ASP to be widely applied in the clinic as a drug for analgesia, antipyretic and heart protection. Besides, it has been indicated in several observational studies and randomized controlled trials that ASP has a chemopreventive effect in malignant tumors. In view of the obvious impact of Wnt/β-catenin imbalance on the occurrence of various malignant tumors, the interaction between ASP and Wnt signaling has become a research hotspot. This study illustrated the understanding of the anti-tumor mechanism of ASP in HCC and delivered some enlightenment for the development of chemoprevention and therapeutic targets of ASP in HCC.

Conclusions

In conclusion, ASP inhibits the proliferation and promotes the apoptosis of HCC cells through the Wnt/β-catenin signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

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