Am J Transl Res 2023;15(12):6779-6785
www.ajtr.org /ISSN:1943-8141/AJTR0153846

*Original Article*

# Aspirin inhibits the biological behavior of gallbladder carcinoma cells by modulating vascular endothelial growth factor

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Received October 9, 2023; Accepted November 30, 2023; Epub December 15, 2023; Published December 30, 2023

**Abstract:** Objective: To elucidate the effect of aspirin (ASP) on the biological behavior of gallbladder carcinoma (GBC) cells and its influence on vascular endothelial growth factor (VEGF) expression. Methods: Cell Counting Kit-8 (CCK-8) assay was performed to determine the effects of ASP on GBC-SD cell proliferation. In addition, Transwell assay and flow cytometry were carried out to observe the role of ASP in GBC-SD cell migration, invasion and apoptosis, respectively. Tumor necrosis factor-α (TNF-α), nuclear factor kappa-B (NF-κB), and VEGF concentrations in GBC-SD cells were examined by enzyme-linked immunosorbent assays (ELISAs). Results: ASP suppressed GBC-SD cell proliferation in a dose-dependent manner, and a concentration ≥ 2 mmol/L could significantly inhibit the migration and invasion of GBC-SD cells and induce apoptosis. In addition, the anticancer effect of ASP in GBC-SD cells may be linked to its inhibition of TNF-α, NF-κB, and VEGF levels. Conclusions: ASP may markedly inhibit GBC-SD cell growth by significantly reducing TNF-α, NF-κB and VEGF expression.

**Keywords:** Aspirin, gallbladder carcinoma, cell biology, VEGF

## Introduction

Gallbladder carcinoma (GBC), a gastrointestinal tumor originating from the biliary tract, is characterized by insidious symptoms, early metastasis, high invasiveness and poor prognosis [1]. Surgical resection, radiotherapy and chemotherapy are the mainstay treatments for GBC; however, they all have their own drawbacks. Most patients have either missed the optimal time for surgical resection due to diagnosis at an advanced stage or disease recurrence and have developed resistance to chemotherapy and radiotherapy after treatment [2, 3]. According to relevant statistics, the 5-year survival of GBC patients is as low as 5% (mean survival: 13.2-19 months), despite constant optimization of treatment strategies for the disease [4]. At present, the pathology of GCB is still poorly understood and it is urgent to formulate new treatment schemes to improve the clinical outcomes of GCB [5]. This study plans to explore the onset and progression of GBC from the perspective of cellular biological behaviors.

Vascular endothelial growth factor (VEGF) is a pro-angiogenic protein that participates in the pathogenesis of GBC and promotes tumor growth and angiogenesis by guiding macrophages to polarize into pro-angiogenic phenotype M2 [6]. It is expressed in up to 80% of GBC patients and affects their survival and prognosis [7]. The study of Ye J et al. [8] has linked VEGF to GBC-SD cell migration in the hypoxic microenvironment, suggesting a certain relationship between VEGF and tumor metastasis in GBC. Aspirin (ASP) has anti-tumor activity in various gastrointestinal malignancies, and its mechanism of action is related to direct inhibition of cyclooxygenase (COX)-2 [9]. There are also case-control studies showing that it can reduce the risk of GBC by 63% and has a certain preventive effect against GBC [10]. ASP has also been found to modulate VEGF expression. Zhang H et al. [11], for instance, reported that ASP significantly reduced VEGF levels in extranodal nasal natural killer/T-cell lymphoma, and the inhibition mechanism was related to its influence on the methylation of Histone

H3K27me3 Antibody (H3K27me3) histone and the dissociation of Specificity Protein 1 (Sp1) on the VEGF promoter. Deng Z et al. [12] also reported the inhibition of ASP on VEGF expression in the rosacea-like skin lesions of mice.

Given the lack of studies investigating the mechanism of action of ASP in GBC onset and progression and its influence on VEGF expression, this study attempts to clarify the underlying mechanism of ASP in GBC treatment, hoping to provide a novel theoretical basis for the treatment and prevention of GBC.

## Materials and methods

### *Cell culture*

The purchased human GBC-SD cell line (BW-6570; Beijing Bovols Bioscience and Technology Co., Ltd.) was cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (BTN19-0190-CMV, Beijing Biolab Technology Co., Ltd.) containing 10% fetal bovine serum (FBS) in an environment of 5% $CO_2$ and 37°C.

### *Cell proliferation*

Cell proliferation was measured strictly following the cell-counting kit (CCK)-8 (Beijing Solarbio Science & Technology Co., Ltd., CK04) manuals. After being inoculated into a 96-well plate (density: 1000 cells/well), cells were treated with 200 μL ASP of various concentrations (0, 1, 2, and 4 mmol/L; Beijing Kangruina Biotech Co., Ltd., A1189) for 24 hours. In addition, GBC-SD was treated with 2 mmol/L ASP for different duration (0, 12, 24, and 48 h). Each well was added with CCK-8 (10 μL) after 2 h of incubation. Finally, the OD value at 450 nm was observed to draw cell proliferation curve.

### *Cell migration and invasion*

Cell migration and invasion were detected by Transwell assay. GBC-SD cells were pretreated with 0 mmol/L ASP (control group) and 2 mmol/L ASP (ASP group) for 24 hours, respectively. After digestion and resuspension, the cells were cultured in a serum-free medium (200 μL; Beijing Zeping Bioscience & Technology Co., Ltd., Lonza medium [435]) and inoculated in the upper chamber. Then, the lower chamber was supplemented with 500 μL medium (20% FBS) and incubated for 24 h. Finally, cells passing through the membrane to the lower chamber were immobilized and stained with 1% crystal violet. Among them, the Transwell chamber in the invasion assay was precoated with 100 μL of 1:6 diluted matrix glue (Wuhan Saios Bioscience and Technology Co., Ltd., ipsSC-CA004) in the cold Dulbecco's Modified Eagle Medium (DMEM), while the migration assay did not involve matrix gel.

### *Apoptosis*

Apoptosis was evaluated using the Annexin V/PI Apoptosis Kit (Proteintech Group, Inc., China, PF00005). GBC-SD cell suspension (2 mL) was cultured in a six-well plate ($1\times10^5$ cells/well) and incubated for 48 hours with 0 (control group) and 2 mmol/L ASP (ASP group), respectively. The harvested cells were washed twice with PBS and stained in the dark for 15 min using a binding buffer (500 μL) comprising 5 μL PI and 5 μL Annexin V. Finally, apoptosis rate was detected and calculated using flow cytometry (Morey Biosciences, Inc., DxFLEX).

### *Enzyme-linked immunosorbent assay (ELISA)*

Tumor necrosis factor-α (TNF-α), nuclear factor kappa-B (NF-κB), and VEGF levels in the supernatant of the cell culture medium in control and ASP groups were determined as instructed by the ELISA kits (Wuhan Fine Biotechnology Co., Ltd., FN-EH030, FN-EH3422, FN-EH0327).

### *Statistical methods*

Data were input into SPSS 23.0 software for analysis, and the results were described in the form of mean ± standard error of the mean (SEM). Comparisons between groups were conducted by the student's t-test or unpaired student's t-test, and those among multiple time points were made by the repeated measures analysis of variance, with the significance identified at $P<0.05$.

## Results

### *ASP inhibited GBC cell proliferation*

GBC cell proliferation in 0 (blank control), 1, 2, and 4 mmol/L groups were analyzed. The blank control and 1 mmol/L groups showed little altered viability of GBC-SD cells (all $P>0.05$), while 2 mmol/L and 4 mmol/L ASP markedly

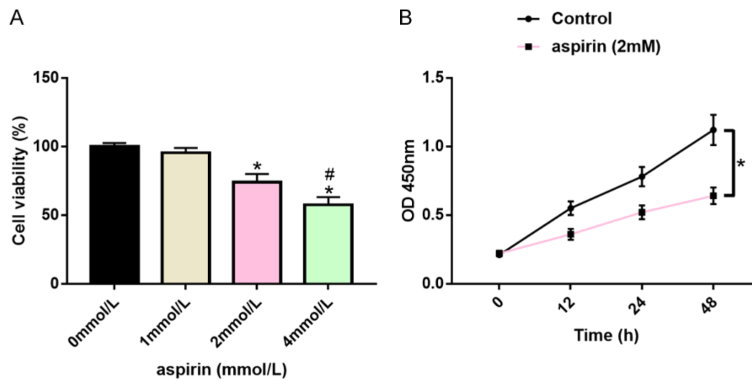


**Figure 1.** Aspirin inhibited gallbladder carcinoma (GBC) cell proliferation. A. Effects of different concentrations of aspirin on GBC-SD cell viability. B. Effects of 2 mmol/L aspirin on GBC-SD cell viability at different time points. Note: *P<0.0 vs. 0 mmol/L group; #P<0.05 vs. 2 mmol/L group.

inhibited GBC-SD cell viability (P<0.05), especially in the 4 mmol/L group (P<0.05). On the other hand, as indicated by CCK-8 assay, 2 mmol/L ASP markedly inhibited GBC-SD cell activity at different time points (P<0.05), so 2 mmol/L ASP was selected for subsequent experiments (**Figure 1**).

*ASP inhibited GBC cell migration and invasion*

As shown by Transwell assay, 2 mmol/L ASP significantly prevented the migration and invasion of GBC-SD cells (all P<0.05, **Figure 2**).

*ASP induced GBC cell apoptosis*

According to flow cytometry, the intervention of 2 mmol/L ASP significantly increased GBC-SD cell apoptosis (P<0.05, **Figure 3**).

*ASP inhibited VEGF expression in GBC cells*

ELISA was performed to determine the impacts of 2 mmol/L ASP on TNF-α, NF-κB, and VEGF levels in GBC-SD cells. The data showed that 2 mmol/L ASP evidently suppressed the levels of these factors (all P<0.05, **Figure 4**).

## Discussion

GBC is a common biliary tract tumor with a high risk of early lymph node infiltration and distant metastasis [13, 14]. Moreover, early-stage GBC patients present with atypical symptoms or abdominal discomfort alone, while advanced patients experience symptoms such as abdominal pain, jaundice and emaciation, which can easily lead to late diagnosis and miss the best opportunity for surgery [15]. Despite the continuous progress made in GBC management, the median survival time of GBC patients remains short, so there is still an urgent need to seek effective drugs [16].

Many researchers have provided new therapeutic directions for the treatment of GBC. For example, Casadei-Gardini A et al. [17] proposed that vitamin D can prolong disease-free survival in patients undergoing cholangiocarcinoma surgery, and that post-chemotherapy oral metformin can improve the prognosis of advanced patients. Yang MH et al. [18] also proposed that Guggulsterone can enhance the antitumor action of gemcitabine by reducing NF-κB activation in GBC cells. ASP used in this study is a pleiotropic anticancer drug that not only exerts a chemoprophylaxis role in multiple tumors, but also prevents tumor metastasis of patients after diagnosis, with the anticancer mechanism related to its inhibition of platelet COX-1 [19]. ASP has also shown clinically to reduce the metastasis risk and exert anticancer actions in colorectal cancer [20]. Moreover, ASP lowers the risk of cholangiocarcinoma by 0.56 times [21]. In this study, we found the dose-dependent inhibitory effect of ASP on cell viability, with significant inhibitory effect at a dose of 2 mmol/L and even more potent inhibiting effect at 4 mmol/L. At different time points, 2 mmol/L ASP was found to validly inhibit GBC-SD cell proliferation compared with the control group. The apoptosis assay results demonstrated the ability of ASP to induce GBC-SD cell apoptosis, further supporting the anti-proliferation action of ASP against GBC-SD cells. In the study of Shi T et al. [22], ASP also significantly inhibited cholangiocarcinoma cell proliferation through its induction of cell cycle arrest. Another study pointed out that ASP prevented proliferation and migration of gastric cancer cells in p53 gene knockout mice, but the specific mechanism has not been clarified.

Metastasis is an important factor threatening the survival of GBC patients and leading to poor

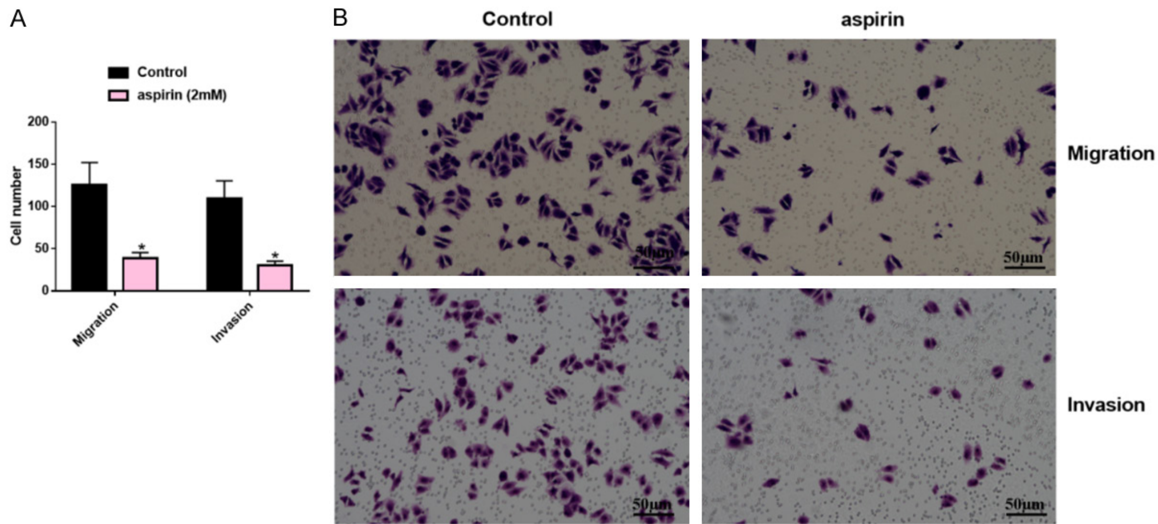


**Figure 2.** Aspirin inhibited migration and invasion of gallbladder carcinoma cells. A: Effect of aspirin on migration and invasion of gallbladder cancer cells; B: Transwell diagram. *P<0.05 vs. control group.

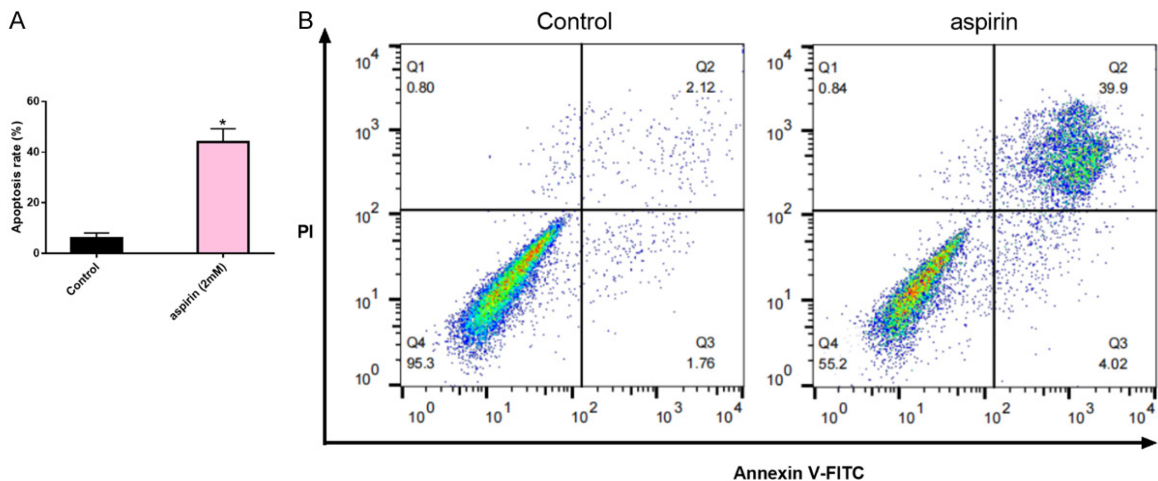


**Figure 3.** Aspirin induced gallbladder carcinoma cell apoptosis. A: Effect of aspirin on gallbladder cancer cell apoptosis; B: Flow cytometry. *P<0.05 vs. control group.

prognosis [23]. The types of GBC metastases include lymph node, vascular, and adjacent liver metastases [24]. Yet, the mechanism of GBC metastases remains poorly understood. In the Transwell assay, ASP exhibited excellent inhibitory effects on GBC-SD cell migration and invasion, suggesting that ASP is conducive to the prevention of GBC metastasis. In the study of Nishimura E et al. [25], ASP reduced the risk of lung metastasis in patients undergoing esophagectomy by preventing neutrophil recruitment. Ying J et al. [26] also proposed that ASP played an effective role in preventing liver metastasis of colorectal cancer, possibly associated with its inhibition of toll-like receptor 4 (TLR4) expression in colorectal cancer cells. In order to further explore the anti-cancer mechanism of ASP, we investigated its influences on TNF-α, NF-κB, and VEGF expression. TNF-α is known to be an important active product of macrophages, a key player in the chronic inflammation mechanism associated with GBC. TNF-α is gradually overexpressed as GBC progresses, and may promote lymph angiogenesis of GBC by promoting the binding of NF-κB to the VEGF-C promoter [27]. NF-κB-related molecular pathways are involved in GBC invasion and lymph node metastasis [28]. Other evidence has

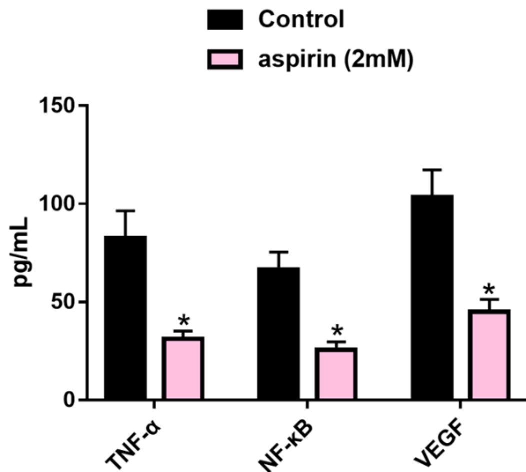


**Figure 4.** Aspirin inhibited VEGF expression in gall bladder carcinoma cells. *P<0.05 vs. control group. VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa-B.

revealed that the TNF-α/NF-κB/VEGF axis is correlated with the angiogenesis and metastasis of colon cancer, and inhibiting this pathway can prevent the aforementioned malignant progression, suggesting that this pathway may also mediate angiogenesis and metastasis of GBC [29]. This study found that ASP significantly down-regulated TNF-α, NF-κB and VEGF levels in GBC-SD, suggesting that the anti-cancer mechanism of ASP in GBC may be related to its inhibition of the TNF-α/NF-κB/VEGF axis.

The limitations of this study: First, IC50 and drug resistance were not analyzed, and supplementation of relevant analysis will be beneficial to enhance the understanding of ASP sensitivity. Second, *in vivo* experiments have not been carried out. If mouse experiments can be supplemented, the impact and corresponding mechanisms of ASP on tumors *in vivo* can be further understood. Finally, the analysis of relevant regulatory mechanisms at the molecular pathway or microRNA (miRNA) level was not included. Supplementing these analyses will help to further understand the mechanisms underlying ASP therapy. The above shortcomings are the focus of future research.

To sum up, ASP can effectively suppress the proliferation, migration and invasion of GBC-SD cells while validly inducing apoptosis, which may be related to its down-regulation of TNF-α, NF-κB and VEGF expression. These findings may provide new insights into GBC treatment and new references for the anti-cancer mechanism of ASP.

## Acknowledgements

I would like to express my gratitude to all those helped me during the writing of this thesis. I acknowledge the help of my colleagues, Minjian Fei and Quan Zhou, as they have offered me suggestion in my academic studies.

## Disclosure of conflict of interest

None.

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