ORIGINAL ARTICLE _____

Effects of aspirin on pancreatic cancer cells PANC-1 and its potential molecular mechanism

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Summary

Purpose: To explore the effects of aspirin on the proliferation and apoptosis of human pancreatic cancer cells and its potential molecular mechanism.

Methods: This study included patients with pancreatic cancer who were divided into experimental group and control group. The cell proliferation ability was detected via cell counting kit-8 (CCK-8) assay and colony forming ability via colony formation assay. In addition, changes in proteins in the phosphatidyl inositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway were assessed using Western blotting, and rescue experiment was conducted to investigate whether aspirin can affect cell proliferation by inhibiting the PI3K/Akt/mTOR signaling pathway.

Results: The results of CCK-8 assay showed that the proliferation rate of PANC-1 cells was decreased in a time- and dose-dependent manner after they were treated with aspirin at different concentrations. Colony formation assay confirmed that cell colony forming ability was significantly re-

duced with the increase in aspirin treatment concentration (p < 0.05). Besides, the apoptosis rate and the number of cells in the experimental group were higher and larger than those in the control group (p<0.05). According to Western blotting results, the protein expressions of PI3K, phosphorylated (p)-Akt and p-mTOR were decreased after aspirin treatment. Rescue experimental results manifested that insulin-like growth factor 1 (IGF-1) supplementation remarkably elevated the expressions of PI3K, p-Akt and p-mTOR compared with phosphate-buffered saline (PBS) supplementation. It was found in CCK-8 assay that IGF-1 supplementation markedly reversed the inhibition of aspirin on the proliferation of PANC-1 cells in comparison with PBS supplementation.

Conclusions: Aspirin inhibits the proliferation and promotes the apoptosis of pancreatic cancer cells by inactivating the PI3K/Akt/mTOR signaling pathway.

Key words: pancreatic cancer, aspirin, PI3K/Akt/mTOR, proliferation, apoptosis

Introduction

Pancreatic cancer, as one of the deadliest and most invasive malignant tumors, is the fourth major cause of cancer-related death in the United States [1]. It is reported that the 5-year survival rate of pancreatic cancer patients is 2-6% [2]. Surgery is the only chance to cure this disease, but most patients miss this chance because of local unresectability or distant metastasis. Worse still, most resected patients will have local or distant recurrence [3]. Some recent anti-inflammatory drug, has been widely used to

studies have revealed that combination chemotherapy aiming at the variant characteristics of pancreatic cancer has enhanced the therapeutic effect [4,5], which broadens our point of view to explore more suitable combinations of anti-tumor drugs or to find targeted drugs aiming at various characteristics for the treatment of pancreatic cancer.

Aspirin (acetylsalicylic acid), a non-steroidal

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relieve pain, fever and inflammation and inhibit platelet function [6]. Recently, increasingly more evidence proves that aspirin is a promising chemopreventive agent and can be used to treat various cancers, including colorectal cancer [7], esophageal cancer [8], gastric cancer [9] and lung cancer [10]. Besides, aspirin can reduce the risks of distant metastasis and long-term death of adenocarcinoma during the incubation period. According to reports, regular application of aspirin after colorectal cancer diagnosis can reduce overall mortality rate of colorectal cancer [11]. Aspirin also shows a therapeutic potential through targeted inhibition on tumor growth and angiogenesis, induction of tumor cell apoptosis, changes in the tumor microenvironment and suppression on tumor metastasis [12,13].

However, less attention has been paid to the role of aspirin in pancreatic cancer. In this study, it was revealed that aspirin blocks the proliferation of pancreatic cancer cells and stimulates their apoptosis. The phosphatidylin-ositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway is a pivotal signaling pathway that regulates the proliferation and apoptosis of tumor cells. This study displayed that the supplementation of the PI3K/Akt/mTOR signaling pathway activator insulin-like growth factor 1 (IGF-1) notably reversed aspirin's inhibition on PANC-1 cancer cell proliferation.

Methods

Materials

Materials included the human pancreatic cancer cell line PANC-1 (American Type Culture Collection) (Manassas, VA, USA), high-glucose Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), pure aspirin (Sigma, St. Louis, MO, USA), cell counting kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan), apoptosis detection kit (Beyotime Biotechnology Co., Ltd., Shanghai, China), IGF-1 (PrimeGene Co., Ltd., Shanghai, China), rabbit antihuman PI3K monoclonal antibody, mouse anti-human phosphorylated (p)-Akt polyclonal antibody and rabbit anti-human p-mTOR polyclonal antibody (Abcam, Cambridge, MA, USA), mouse anti-human β -actin antibody (Sigma, St. Louis, MO, USA), FACScan flow cytometer (BD, Franklin Lakes, NJ, USA) and fluorescence microscope (Olympus, Tokyo, Japan).

Cell culture and drug preparation

Pancreatic cancer cell line was cultured in high-glucose DMEM containing 10% FBS and 100 g/mL streptomycin, and placed in a humid incubator with 5% CO_2 at 37°C. Then pure aspirin was dissolved in absolute ethyl alcohol, whose concentration was prepared into 1000× firstly and then diluted into 1× with DMEM.

Detection of cell proliferation via CCK-8 assay

PANC-1 cells in the logarithmic growth phase were taken to prepare a single cell suspension at a concentration of 2×10^4 /mL. Thereafter, 100 µL of the suspension was added to each well and inoculated into 96-well plates. After 12 h of culture in the incubator, the original medium was replaced with aspirin-containing medium, whose final concentration was made into 0, 0.5, 1, 2, 4 mmol/L, respectively. Six multiple wells were set at each point in each concentration group. Subsequently, 10 µL of the CCK-8 solution was added to each well for staining after 24, 48 and 72 h of continuous drug treatment. After incubation in the incubator at 37°C for 3 h, the absorbance at 450 nm was measured using a microplate reader.

Colony formation assay

PANC-1 cells were inoculated into 6-well plates at a density of 300 cells/well and cultured in DMEM containing 10% FBS and aspirin at the corresponding concentration for 2 weeks, and the medium was replaced once every 4 days. Fourteen days later, cell colonies were fixed with methanol/acetic acid solution (10% methanol, 10% acetic acid, v/v), stained with crystal violet (1% w/v) and counted manually using a stereo microscope. Three replicate wells were set in each concentration group.

Detection of cell apoptosis rate via flow cytometry

PANC-1 cells were inoculated into 6-well plates (10⁵ cells/well), and the original medium was replaced with a medium containing 1 mmol/L aspirin after the cells adhered to the wall for 12 h. The other cells whose culture medium was replaced with a medium containing no aspirin were taken as the control group. Cell apoptosis was detected after continuous culture for 48 h, and the cells were digested with trypsin without EDTA (ethylenediaminetetraacetic acid) and collected after centrifugation. After that, the cells were gently re-suspended with phosphate-buffered saline (PBS) and counted. Then, 10⁵ re-suspended cells were taken, centrifuged, added with FITC binding solution firstly and then with Annexin V-FITC (fluorescein isothiocyanate), and mixed gently, followed by the addition of propidium iodide (PI) staining solution and gentle mixing. Finally, cell apoptosis was detected by flow cytometry after incubation at room temperature in the dark for 15 min.

Determination of the number of apoptotic cells through terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After treatment with the added drug for 48 h, cells were collected and made into single cell PBS suspension. Then, an appropriate amount of suspension was absorbed and dropped on the glass slide, immersed in 4% paraformaldehyde and fixed at 4°C for 25 min. According to the operation instructions of TUNEL cell apoptosis detection kit, TUNEL-positive cells were observed and counted using a fluorescence microscope.

Detection via Western blotting

Total proteins were extracted from PANC-1 cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA) was applied to quantify the total protein concentration. Fifty µg of total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane. After that, the membrane was slowly sealed with 5% skim milk powder prepared by trisbuffered saline with Tween 20 (TBST) in a shaking table at room temperature for 1 h and the proteins were incubated with primary antibody at 4°C overnight. Subsequently, the membrane was rinsed with TBST and the proteins were incubated with the secondary antibody at room temperature for 1 h, followed by washing with TBST again. Ultimately, the target protein was examined on the electrochemiluminescence (ECL) color development system.



Figure 1. Effect of aspirin on the proliferation of human pancreatic cancer PANC-1 cells detected *via* CCK-8. Compared with untreated cells, PANC-1 cells treated with 4 mM aspirin had 40% proliferation inhibition rate (p<0.03).

Statistics

The experimental data were analyzed by SPSS 19.0 software (IBM, Armonk, NY, USA), expressed as mean±standard deviation, and detected using t-test. P<0.05 suggested that the difference was statistically significant.

Results

Aspirin suppressed the proliferation of pancreatic cancer cells

First, CCK-8 was used to detect the effect of aspirin on the proliferation of human pancreatic cancer PANC-1 cells. After PANC-1 cells were treated with 0.5, 1, 2 and 4 mM aspirin, the cell viability was measured at 24, 48 and 72 h, respectively. The growth curve in Figure 1 displayed that the cell proliferation rate after aspirin treatment was decreased in a time- and dose-dependent manner. Compared with untreated cells, PANC-1 cells treated with 4 mM aspirin had a proliferation inhibition rate of 40% at 24 h (p<0.05), and the inhibitory effect of aspirin became more significant at 72 h. It can be obviously seen that aspirin is able to block the proliferation of human pancreatic cancer cells.

Aspirin inhibited the colony forming ability of pancreatic cancer cells

Next, the effect of aspirin on the colony forming ability of human pancreatic cancer PANC-1



Figure 2. Effect of aspirin on the colony forming ability of human pancreatic cancer PANC-1 cells. With increase of aspirin dosage the cell colony forming ability was significantly reduced (*p<0.05).



Figure 3. A: Effect of aspirin on the apoptosis rate of human pancreatic cancer PANC-1 cells examined via flow cytometry (*p<0.05). B: Effect of aspirin on the number of apoptotic human pancreatic cancer PANC-1 cells determined by TUNEL assay (*p<0.05).

cells was further verified through the colony formation assay which showed that with the increase in aspirin treatment concentration, the cell colony forming ability was obviously reduced (p<0.05) (Figure 2).

Aspirin induced apoptosis of pancreatic cancer cells

The effect of aspirin on the apoptosis rate of PANC-1 cells was analyzed by flow cytometry. As shown in Figure 3A, the apoptosis rate in the experimental group was notably higher than that in the control group (p<0.05). Meanwhile, TUNEL assay was employed to determine the effect of aspirin on the apoptosis of PANC-1 cells. The results (Figure 3B) manifested that compared with the control group, aspirin treatment evidently increased the number of apoptotic PANC-1 cells (p<0.05). Therefore, the above findings indicate that aspirin can induce apoptosis of pancreatic cancer cells.

Aspirin repressed the PI3K/Akt/mTOR signaling pathway

The PI3K/Akt/mTOR pathway is an important signaling pathway that regulates the proliferation and apoptosis of tumor cells. In order to determine the effect of aspirin, the proteins in this pathway were tested by Western blotting after aspirin (2 mM) treatment. According to the results (Figure 4), mTOR signaling pathway was further evaluated



Figure 4. Influence of aspirin on the protein expression level in the PI3K/Akt/mTOR pathway measured via Western blotting. The aspirin treatment inhibited significantly the protein expression level of PI3K, p-Akt and p-mTOR

aspirin treatment could remarkably inhibit the protein expression levels of PI3K, p-Akt and p-mTOR, suggesting that aspirin is capable of blocking the PI3K/Akt/mTOR signaling pathway in pancreatic cancer cells.

Activation of the PI3K/Akt/mTOR pathway reversed the inhibition of aspirin on the proliferation of PANC-1 cells

The activation of aspirin on the PI3K/Akt/



Figure 5. Activation of the PI3K/Akt/mTOR pathway reversed the influence of aspirin on the proliferation of PANC-1 cells. **A:** The results demonstrated that IGF-1 supplementation markedly elevated the expressions of PI3K, p-Akt and p-mTOR compared with PBS supplementation. **B:** CCK-8 assay revealed that IGF-1 supplementation significantly promoted the proliferation of PANC-1 cells in comparison with PBS supplementation (p=0.05).

to confirm whether aspirin inhibits cell proliferation by inhibiting this pathway. IGF-1, as an activator of the PI3K/Akt/mTOR signaling pathway, can reverse the inhibitory effect of aspirin on the proliferation of PANC-1 cells. Firstly, the changes in the PI3K, p-Akt and p-mTOR expression levels in PANC-1 cells supplemented with IGF-1 or PBS after aspirin (2 mM) treatment were detected using Western blotting. The results demonstrated that IGF-1 supplementation markedly elevated the expressions of PI3K, p-Akt and p-mTOR compared with PBS supplementation (Figure 5A). CCK-8 assay revealed that IGF-1 supplementation significantly promoted the proliferation of PANC-1 cells in comparison with PBS supplementation (Figure 5B).

Discussion

Pancreatic cancer, a malignant tumor of the digestive system, has a high grade of malignancy and is difficult to diagnose and treat. Approximately 90% of pancreatic cancer derives from ductal adenocarcinoma of the glandular epithelium. Over the past few decades, the incidence rate of pancreatic cancer has risen rapidly in China [14]. Despite the currently improved diagnosis and treatment of pancreatic cancer, its prognosis is still unsatisfactory mainly due to non-specific symptoms, lack of curative treatment and delayed diagnosis [15]. Therefore, it is necessary to seek drugs for targeted treatment of pancreatic cancer in order to provide more reliable and precise treatment options.

Aspirin can reduce the risks of distant metastasis and long-term death of adenocarcinoma, so it is inferred to have a therapeutic potential in pancreatic cancer. As expected, aspirin treatment obviously suppresses the proliferation of PANC-1 cells in a dose- and time-dependent manner. Flow cytometry and TUNEL results verified that aspirin could induce apoptosis of pancreatic cancer cells. In 2012, a research revealed that the daily continuous application of low-dose aspirin can lower the risk of distant metastasis of cancer [16]. Several proliferation inhibition mechanisms of aspirin include the inhibition of the Wnt/ β -catenin and PI3K/Akt signal transduction and regulation on the activation of Bax and Bcl-2 [17,18]. Sun et al [19] discovered that aspirin represses mTORC1 signal transduction in AMPK-dependent and non-dependent ways, and blocks the AMPK-TSC pathway to inhibit the mTORC1 activity. In the meantime, it directly breaks the mTOR-raptor interaction. Moreover, the combination of aspirin and sorafenib shows synergistic effects by inhibiting mTORC1 signal transduction as well as PI3K/Akt and MAPK/ERK pathways.

The PI3K/Akt/mTOR pathway is a crucial signaling pathway that regulates the proliferation and apoptosis of tumor cells. The PI3K/Akt pathway is inappropriately activated in different types of cancers. PI3K/Akt can be activated through two main mechanisms, namely, the activation of specific nodes and receptor tyrosine kinase in the pathway. Therefore, understanding the activation mechanism of PI3K is a key for developing effective PI3K inhibitors [20]. It is reported that abnormalities in the PI3K/Akt/mTOR signaling pathway exert effects on tumor cells, and even can be constitutively activated to promote the growth and proliferation of tumor cells [21]. Aspirin can break the positive feedback between KRAS and prostaglandin E2 (PGE2) and suppress KRAS signal transduction. KRAS up-regulates PTGS2 expression and increases the production of PGE2 through the MAPK and PI3K/Akt pathways, while

PGE2 reversely elevates the level of GTP binding to RAS [22]. Next, the anti-tumor effect of aspirin in pancreatic cancer cells was tested according to the changes in PI3K, p-Akt and p-mTOR expressions to evaluate changes in the PI3K/Akt/mTOR signaling pathway. In recent reports, a large number of studies have indicated that the mechanism of silencing the PI3K/Akt/mTOR signaling pathway through traditional Chinese medicine is related to anti-cancer effect [23-25]. It was discovered that IGF-1 activated the PI3K/Akt/mTOR signaling pathway so as to reverse aspirin's inhibition on the proliferation of pancreatic cancer cells. The above findings suggest that aspirin plays an anti-cancer role in pancreatic cancer cells by adjusting the PI3K/Akt/mTOR signaling pathway.

Conclusions

To sum up, it is confirmed that aspirin inhibits the proliferation and stimulates the apoptosis of pancreatic cancer cells by inactivating the PI3K/ Akt/mTOR signaling pathway, which is conducive to expanding the application prospect of aspirin in resisting cancers. However, further investigating the specific mechanism of its action and its ideal anti-cancer dose in clinical environment is necessary, so as to kill tumors with the highest efficiency and reduce side effects, which will surely have greater clinical significance.

Conflict of interests

The authors declare no conflict of interests.

References

- 1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.
- 2. Tentes AA, Pallas N, Karamveri C, Kyziridis D, Hristakis C. Cytoreduction and HIPEC for peritoneal carcinomatosis of pancreatic cancer. J BUON 2018;23:482-7.
- Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet 2011;378:607-20.
- 4. Sleightholm RL, Neilsen BK, Li J et al. Emerging roles of the CXCL12/CXCR4 axis in pancreatic cancer progression and therapy. Pharmacol Ther 2017;179:158-70.
- 5. Lee J, Kim DH, Kim JH. Combined administration of naringenin and hesperetin with optimal ratio maximizes the anti-cancer effect in human pancreatic cancer via down regulation of FAK and p38 signaling pathway. Phytomedicine 2019;58:152762.
- 6. Cortellini G, Caruso C, Romano A. Aspirin challenge and desensitization: how, when and why. Curr Opin Allergy Clin Immunol 2017;17:247-54.
- 7. Giampieri R, Restivo A, Pusceddu V et al. The Role of Aspirin as Antitumoral Agent for Heavily Pretreated Patients With Metastatic Colorectal Cancer Receiving Capecitabine Monotherapy. Clin Colorectal Cancer 2017;16:38-43.
- Ararat E, Sahin I, Altundag K. Mechanisms behind the aspirin use and decreased breast cancer incidence. JBUON 2011;16:180.
- 9. Hao Q, Gong H, Zong H et al. Aspirin use improves the biochemical control of prostate cancer in Chinese men. JBUON 2018;23:1803-8.
- 10. Lim WY, Chuah KL, Eng P et al. Aspirin and non-aspirin non-steroidal anti-inflammatory drug use and risk of lung cancer. Lung Cancer 2012;77:246-51.
- 11. Ye XF, Wang J, Shi WT, He J. Relationship between aspirin use after diagnosis of colorectal cancer and patient

survival: a meta-analysis of observational studies. Br J Cancer 2014;111:2172-9.

- 12. Zelenay S, van der Veen AG, Bottcher JP et al. Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. Cell 2015;162:1257-70.
- 13. Ergun Y, Ozdemir NY, Guner EK et al. Comparison of Gemcitabine monotherapy with Gemcitabine and Cisplatin combination in metastatic pancreatic cancer: a retrospective analysis. J BUON 2018;23:116-21.
- 14. Shi W, Lu Y, Gong R, Sun JJ, Liu G. Serum miR-629 is a novel molecular marker for diagnosis and the prognosis of pancreatic cancer. Eur Rev Med Pharmacol Sci 2018;22:5187-93.
- 15. El HB, Li PG, Liu D et al. Pharmacogenetics of treatments for pancreatic cancer. Expert Opin Drug Metab Toxicol 2019;15:437-47.
- 16. Chen Z, Li W, Qiu F et al. Aspirin cooperates with p300 to activate the acetylation of H3K9 and promote FasL-mediated apoptosis of cancer stem-like cells in colorectal cancer. Theranostics 2018;8:4447-61.
- 17. Wang Y, He G, Wang F et al. Aspirin inhibits adipogenesis of tendon stem cells and lipids accumulation in rat injury tendon through regulating PTEN/PI3K/AKT signalling. J Cell Mol Med 2019;23:7535-44.
- Ding JH, Yuan LY, Huang RB, Chen GA. Aspirin inhibits proliferation and induces apoptosis of multiple myeloma cells through regulation of Bcl-2 and Bax and suppression of VEGF. Eur J Haematol 2014;93:329-39.
- 19. Sun D, Liu H, Dai X et al. Aspirin disrupts the mTOR-Raptor complex and potentiates the anti-cancer activities of sorafenib via mTORC1 inhibition. Cancer Lett 2017;406:105-15.
- 20. Ebrahimi S, Hosseini M, Shahidsales S et al. Targeting the Akt/PI3K Signaling Pathway as a Potential Thera-

peutic Strategy for the Treatment of Pancreatic Cancer. Curr Med Chem 2017;24:1321-31.

- 21. Niu NK, Wang ZL, Pan ST et al. Pro-apoptotic and proautophagic effects of the Aurora kinase A inhibitor alisertib (MLN8237) on human osteosarcoma U-2 OS and MG-63 cells through the activation of mitochondriamediated pathway and inhibition of p38 MAPK/PI3K/ Akt/mTOR signaling pathway. Drug Des Devel Ther 2015;9:1555-84.
- 22. Jiang MJ, Dai JJ, Gu DN, Huang Q, Tian L. Aspirin in pancreatic cancer: chemopreventive effects and therapeutic potentials. Biochim Biophys Acta 2016;1866:163-76.
- 23. Lin YT, Wang HC, Hsu YC, Cho CL, Yang MY, Chien CY. Capsaicin Induces Autophagy and Apoptosis in

Human Nasopharyngeal Carcinoma Cells by Downregulating the PI3K/AKT/mTOR Pathway. Int J Mol Sci 2017;18:1343.

- 24. Lin YT, Wang HC, Chuang HC, Hsu YC, Yang MY, Chien CY. Pre-treatment with angiotensin-(1-7) inhibits tumor growth via autophagy by downregulating PI3K/ Akt/mTOR signaling in human nasopharyngeal carcinoma xenografts. J Mol Med (Berl) 2018;96:1407-18.
- 25. Yanwei L, Yinli Y, Pan Z. Traditional Herbal Formula NPC01 Exerts Antiangiogenic Effects through Inhibiting the PI3K/Akt/mTOR Signaling Pathway in Nasopharyngeal Carcinoma Cells. Evid Based Complement Alternat Med 2018;2018:5291517.