Translational Oncology & Therapeutics

Original Article

Exploring The Mechanism Of Action Of The Combination Of Oleanolic Acid And Aspirin In The Treatment Of Colorectal Cancer

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- Received Date: 04 May 2023
- Accepted Date: 27 May 2023
- Published Date: 29 May 2023

Keywords

oleanolic acid, aspirin, colorectal cancer, experimental validation

Abstract

Colorectal cancer (CRC) is one of the common malignancies, and the drug resistance and severe toxicity generated by long-term chemotherapy in colorectal cancer patients severely limit the efficiency of chemotherapy for colorectal cancer. Aspirin is currently one of the most promising chemopreventive drugs for CRC, but its long-term use is prone to side effects such as gastrointestinal bleeding, which limits its clinical application. Oleanolic acid is an effective anti-tumor drug, which can inhibit the proliferation of many kinds of colorectal cancer cells in colorectal cancer. Therefore, this study was designed to investigate the potential mechanism of action of the combination of oleanolic acid and aspirin against colorectal cancer through in vitro experiments. The results of cellular assays showed that oleanolic acid and aspirin inhibited the proliferation of HT-29 and HCT-116 cells, and oleanolic acid dose-dependently inhibited the expression of PTGS2 in HT-29 cells and enhanced the expression of PTGS2 and inhibited the degradation of IκBα in colorectal cancer cells by aspirin. This study reports for the first time the anti-novel combination of oleanolic acid and aspirin against colorectal cancer. It provides a new direction for the development of new drugs for the treatment of colorectal cancer.

Introduction

Colorectal cancer is a highly prevalent cancer worldwide, with the latest research data showing that it has the 3rd highest incidence and 2nd highest mortality rate of cancer worldwide [1]. The traditional treatment modalities for colorectal cancer include surgery, chemotherapy and radiotherapy. Due to the insidious nature of the disease and unclear symptoms, most patients are clinically diagnosed as advanced colorectal cancer and can only receive chemotherapy. In addition, even patients with colorectal cancer who undergo radical resection should receive chemotherapy and radiation therapy after surgery. Drug resistance and severe toxicity severely limit the efficiency of chemotherapy for colorectal cancer [2,3]. Aspirin is a nonsteroidal anti-inflammatory drug that can be used in anti-inflammatory and anti-rheumatic therapy and has antipyretic and analgesic effects [4]. Studies have confirmed that aspirin has protective properties for patients with colorectal cancer and can effectively reduce the risk of tumor development, improve the prognosis of colorectal cancer, and reduce the morbidity and mortality of colorectal cancer [5, 6]. Encouragingly, a large number of natural compounds have been identified in human cancer research as potential anticancer agents with cytotoxic and anti-proliferative activities [7].

Oleanolic acid (OA: 3β-hydroxy-olea-12-en-28-oic acid; Figure 1) is a pentacyclic triterpenoid widely found in different medicinal and edible plants, which was first discovered and isolated from Lignanaceae by the British chemist FB Powers, and its structure was later determined by Ruzicka [8]. It has pharmacological effects such as antioxidant [9], anti-inflammatory [10], anti-ulcer [11], antibacterial [12], and anti-viral [13], as well as antagonistic effects on a variety of tumors [14, 15]. Studies have shown that OA can induce apoptosis in a variety of tumor cells, such as colorectal cancer [16], glioma [17], lung cancer, pancreatic cancer [18], cervical cancer [19], ovarian cancer [20] cells, and many other tumor cells with anti-proliferative effects. Therefore, OA is a promising therapeutic agent for colorectal cancer.

Due to the anti-colorectal cancer activity of oleanolic acid and aspirin, we proposed the hypothesis that the combination of oleanolic acid and aspirin could enhance the anti-colorectal cancer effect of aspirin. The aim of this study was to explore the effect of oleanolic acid on the biological behavior of colorectal cancer cell lines HT-29 and HCT-116 cells, and to explore the mechanism of its anti-colorectal cancer effect in combination with aspirin

Citation: Lin S, Chen Y, Huang F, Lin M, Xu L. Exploring The Mechanism Of Action Of The Combination Of Oleanolic Acid And Aspirin In The Treatment Of Colorectal Cancer. Transl Oncol Ther. 2023; 1(1):1-6.

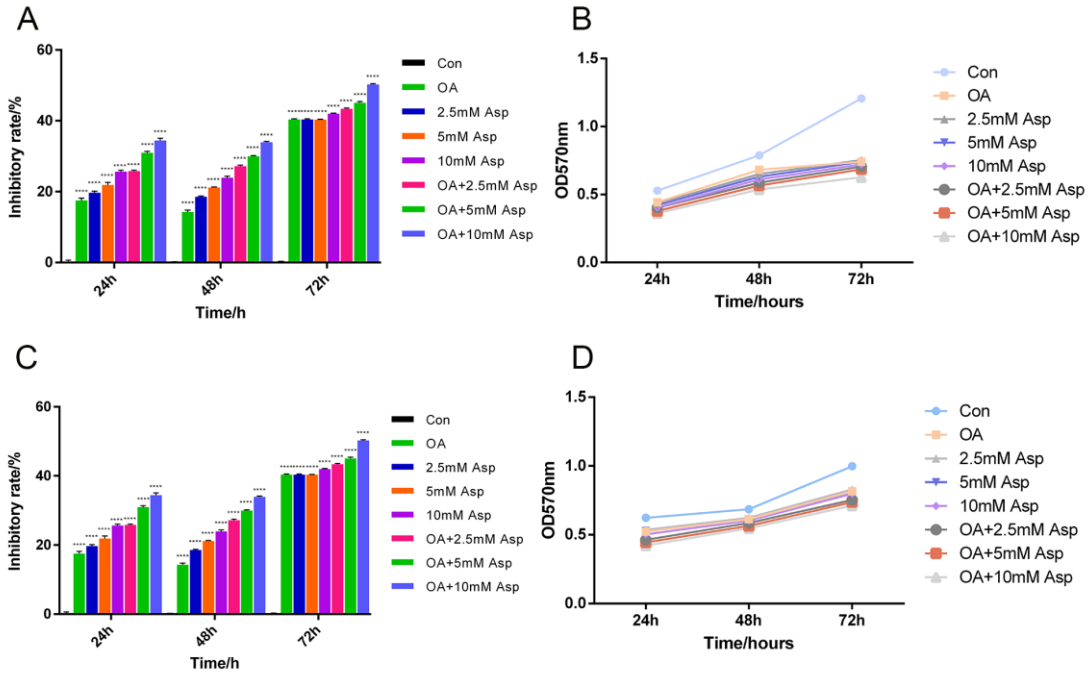

Figure 1. Oleanolic acid enhances the inhibition of proliferation of colorectal cancer cells by aspirin.
A and B: HT-29; C and D: HCT-116

by western blot and RT-qPCR, in order to provide a theoretical basis for improving the anti-tumor effect of aspirin and reducing the side effects such as bleeding caused by aspirin.

Materials and methods

Cell culture

Colorectal cancer cell line HT-29 and HCT-116 cells was purchased from Wuxi Xinrun Biological Co., Ltd (Wuxi, China). HT-29 and HCT-116 cells were cultured in DMEM high glucose medium containing 10% fetal bovine serum, 2 mM L-glutamic acid, non-essential amino acids and sodium pyruvate, at 37°C, 5% CO_2 in a constant temperature incubator. When the cells grow to 80-90% confluence, remove the cells from the cell culture incubator, place them in the ultra-clean bench, aspirate and discard the medium, PBS wet wash, add 0.25% trypsin to digest for 1-2 min, add appropriate amount of complete medium when the cells become round and not yet floating and repeatedly blow to make the cells completely and uniformly suspended, divide the cell suspension evenly into culture dishes according to 1 pass 3 and place them in the incubator. The cells were incubated for 2-3 days.

Cell viability assay

The MTT assay was used to detect the growth inhibitory effects of oleanolic acid on different tumor cells. Four treatments methods were set up: negative control group, oleanolic acid (10 μM) group, aspirin (2.5, 5, 10 mM) group, and co-drug group . 4 groups of cells were grown in 96-well culture plates at a density of 5×10^3 cells. 10 μM oleanolic acid was added to the oleanolic acid mono- and co-drug groups, and 2 h later different concentrations (2.5, 5, 10 mM) of aspirin were added to the aspirin mono- and co-drug groups. Three replicate wells were set up for each treatment. After incubation in a constant temperature incubator at 37°C, 5% CO_2 for 24, 48 and 72 hours. The MTT storage solution at a final concentration of 0.5 mg/ml was added to each well at 10 μl per well, and after 4 hours in the incubator, the medium was completely removed and 100 μl DMSO was added to dissolve the crystals. The culture plate was placed in an enzyme marker, and the absorbance value was detected at 570 nm to calculate the cell proliferation inhibition rate. Cell proliferation inhibition rate (%) = (average OD value of control wells - average OD value of test wells)/(average OD value of control wells - average OD value of blank wells) × 100%.

Detection of related protein expression by Western blot

Detection of PTGS2 expression in colorectal cancer HT-29 cells by different concentrations of oleanolic acid

HT-29 cells were grown in 6-well plates, treated with different concentrations (0, 5, 10, 20 μM) of oleanolic acid, incubated for 48 h and then total cellular proteins were extracted with RIPA rapid lysate, and protein concentrations were quantified using the BCA protein analysis kit. The proteins in the cells were separated by SDS-PAGE separation gel and transferred to PVDF membranes, which were closed in 5% skim milk for 1 h, washed three times in TBST, and incubated with PTGS2 monoclonal antibody (1:1000; Affinity). And GAPDH was used as a loading control. Then, the immunoblots were incubated with HPR-coupled goat anti-rabbit secondary antibody (Solebro) for 1 h at 37°C, stained with enhanced ECL chemiluminescent substrate, and visualized by Tanon 5200 chemiluminescent imaging system. The gray density of each protein band was normalized to the gray density of GAPDH. Each assay was repeated in triplicate.

Detection of the effect of oleanolic acid on the expression of COX-2 in aspirin-resistant colorectal cancer cells

HT-29 cells were grown in 6-well plates, negative control group, oleanolic acid (10 μM) single drug group, aspirin (10 mM) single drug group, and combination drug group, and cells were treated according to these four treatments, and total cellular protein was extracted by RIPA rapid lysis solution after 48 h of incubation, and protein concentration was quantified using BCA protein analysis kit. The proteins in the cells were separated by SDS-PAGE separation gel and transferred to PVDF membranes, which were closed in 5% skim milk for 1 h, washed 3 times

in TBST, and incubated with rabbit anti-human need to confirm COX-2 monoclonal antibody (1:1000; Affinity). And GAPDH was used as a loading control. Then, the immunoblots were incubated with HPR-coupled goat anti-rabbit secondary antibody for 1h at 37°C, stained with enhanced ECL chemiluminescent substrate and visualized by Tanon 5200 chemiluminescent imaging system. The gray density of each protein band was normalized to the gray density of GAPDH. Each assay was repeated in triplicate.

Detection of the effect of oleanolic acid-enhanced aspirin on TNF-α-induced degradation of IκBα

HT-29 cells were grown in 6-well plates, and negative control group, oleanolic acid (10 μM) single drug group, aspirin (10 mM) single drug group, and combination drug group were set up. cells were treated according to these four treatments, and incubated for 2 h and then treated with 3 ng/ml TNF-α and continued to incubate for 30 min. total cellular protein was extracted using RIPA rapid lysis solution, and protein was quantified using BCA protein The protein concentration was quantified using the BCA protein analysis kit. The proteins in the cells were separated by SDS-PAGE separation gel and transferred to PVDF membranes, which were closed in 5% skim milk for 1h, washed three times in TBST, and incubated with rabbit anti-human required confirmation of IκBα monoclonal antibody (1:1000; Affinity). And GAPDH was used as a loading control. Then, immunoblots were incubated with HPR-coupled goat anti-rabbit secondary antibody for 1 h at 37°C, stained with enhanced ECL chemiluminescent substrate, and visualized by Tanon 5200 chemiluminescent imaging system. The gray density of each protein band was normalized to the gray density of GAPDH. Each assay was repeated in triplicate.

RT-PCR assay

HT-29 cells were grown in well plates, negative control group, oleanolic acid (10 μM) single drug group, aspirin (10 mM) single drug group, and combination drug group were set up, and HT-29 cells were treated according to these four treatments. After incubation for 48 h, total cellular RNA was extracted with Trizol, and RNA samples were transcribed into cDNA, followed by real-time quantitative using specific primers (Table 1) polymerase chain reaction with GAPDH as an internal control. PCR reaction conditions were: pre-denaturation at 95°C for 5 min; denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 30 s, and terminal extension at 72°C for 7 min, for a total of 40 cycles. The effect of different treatments on the mRNA levels of COX-2 was examined by electrophoresis on agarose gels and collecting images. Statistical analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Statistical analysis was performed using SPSS 21.0 software, and all test data were described as mean ± standard deviation (SD), one-way ANOVA was used for multiple group comparisons, and t-test was used for comparison of two groups. $p<0.05$ indicates that the difference is statistically significant. GraphPad Prism 6.0 software was used for image processing.

Table 1. *Primer sequences.*

Name	Primer sequences
COX-2 (PTGS2)	5'-GGGAAGCCTTCTCTAACCTC-3'
	5'-CTGCTTGTCTGGAACAACTG-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'
	5'-TCCACCACCCTGTTGCTGTA-3

Results

Enhanced inhibition of colorectal cancer cell proliferation by aspirin with oleanolic acid

HT-29 colorectal cancer cell lines were selected, and four treatments were set up: negative control group, oleanolic acid (10 μM) monotherapy group, aspirin (2.5, 5, 10 mM) monotherapy group, and combined drug (oleanolic acid 10 μM + aspirin (2.5, 5, 10 mM)) group. The effects of oleanolic acid and aspirin on the proliferation of these 2 colorectal cancer cell lines were examined by MTT method. The results showed that the effects of different treatments on the proliferation of colorectal cancer HT-29 and HCT-119 cells were examined at 24, 48 and 72 h of treatment, respectively. The results showed (Figure 1) that the inhibition rate of oleanolic acid applied alone on the value-added of HT-29 and HCT-119 cells ranged from 11.02-40.82%, and its inhibitory effect was enhanced with the prolongation of incubation time, and the highest proliferation inhibition rate reached 40.82% for the treatment of HT-29 cells for 72h. The inhibition rate of proliferation of the two cell types by different concentrations of aspirin alone ranged from 9.14-42.20%, and the strongest inhibition of proliferation of HT-29 cells was achieved by incubating the cells for 72 h after treatment, reaching 42.20%. The inhibition rate of the combined application on the proliferation of these two kinds of cells reached 15.25-50.59%, and the inhibitory effect on the proliferation of HT-29 cells was the strongest after 72 hours of incubation, reaching 50.59%. The results showed that the inhibition of HT-29 cell proliferation by oleanolic acid, aspirin and the combination was time- and concentration-dependent, while the inhibition rate of HCT-116 cell proliferation was maximized at 24 h. The inhibition rate of HCT-116 cell proliferation increased with the increase of the concentration. This suggested that oleanolic acid could enhance the inhibitory effect of aspirin on the proliferation of colorectal cancer cells..

Inhibition of PTGS2 expression by oleanolic acid in colorectal cancer HT-29 cells

Colorectal cancer HT-29 cells with high PTGS2 expression were selected to detect the inhibition of PTGS2 expression by oleanolic acid. HT-29 cells were grown in 6-well plates and treated with different concentrations of oleanolic acid. The expression of PTGS2 in the cells was detected by western blot assay. The results showed (Figure 2) that oleanolic acid could dose-dependently inhibit the expression of PTGS2 in the HT-29 cell line.

Oleanolic acid enhances the inhibitory effect of aspirin on PTGS2 expression

To detect the effect of oleanolic acid and aspirin on the expression of PTGS2 in HT-29 cells. The cells were grown in 6-well plates, and HT-29 cells were treated with the four treatments mentioned above. The expression of PTGS2 in HT-29 cells was detected by western blot and RT-PCR. The results showed that oleanolic acid enhanced the inhibition of PTGS2 expression in HT-29 cells by aspirin at the protein and mRNA (Figure 3) levels after treatment of HT-29 cells for 48h. This suggests that the enhancement of aspirin inhibition of colorectal cancer cell proliferation by combined treatment with oleanolic acid and aspirin may be achieved through inhibition of COX-2 expression.

Oleanolic acid enhances the inhibition of TNF-α-induced IκBα degradation by aspirin

Since the nuclear entry and transcriptional activity of NF-κB can regulate the expression of PTGS2, in this study, HT-29 cells were grown in 6-well plates, and after the above four treatments for 2 h, the cells were treated with 3 ng/mL of TNF-α for 30 min, and then the degradation of IκBα in the cells was detected by western blotting as a way to reflect the effect of different treatments on NF-κB activity. The results showed that oleanolic acid enhanced the inhibition of the

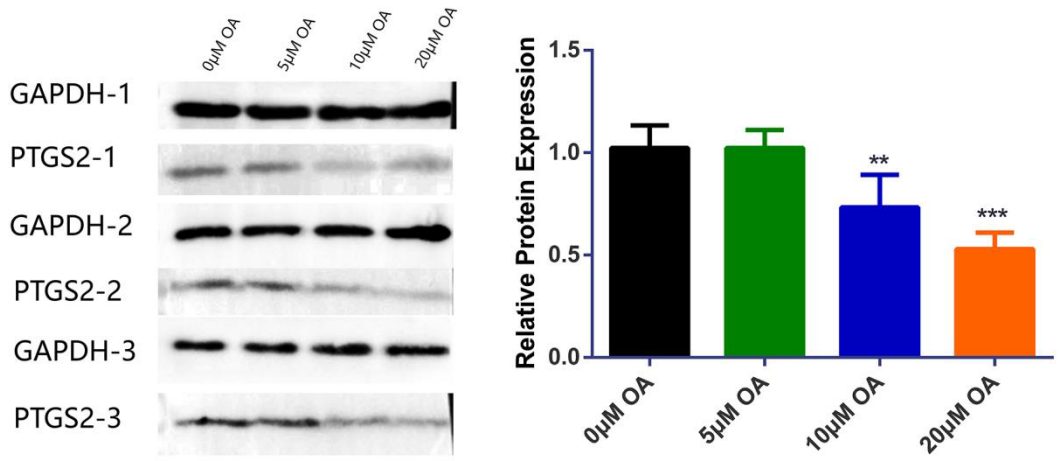

Figure 2. ***Oleanolic acid inhibits the expression of PTGS2 in colorectal cancer HT-29 cells.***
A: Electrophoresis of PTGS2 protein in HT-29 cells, GAPDH was used as a loading control. B: Quantification of the corresponding grayscale values of the western blotting. Western blots were performed in triplicate. Each bar represents the mean ± SD from three independent assays. **P < 0:01 ; *** P < 0:001.

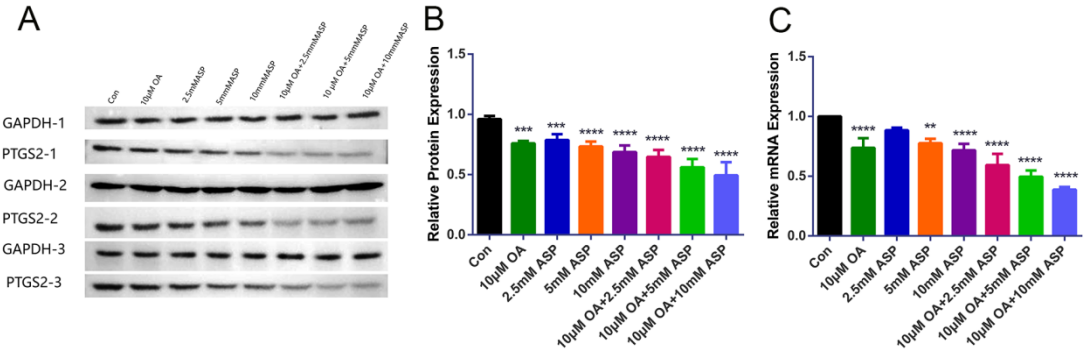

Figure 3. ***Oleanolic acid enhances the inhibition of PTGS2 expression by aspirin in HT-29 cells.***
A and B: Western blot assay to detect the inhibition of PTGS2 expression in HT-29 cells by oleanolic acid-enhanced aspirin; C: RT-PCR assay to detect the inhibition of PTGS2 expression in HT-29 cells by oleanolic acid-enhanced aspirin. Western blots were performed in triplicate. Each bar represents the mean ± SD from three independent assays. **P < 0:01 ; *** P < 0:001.

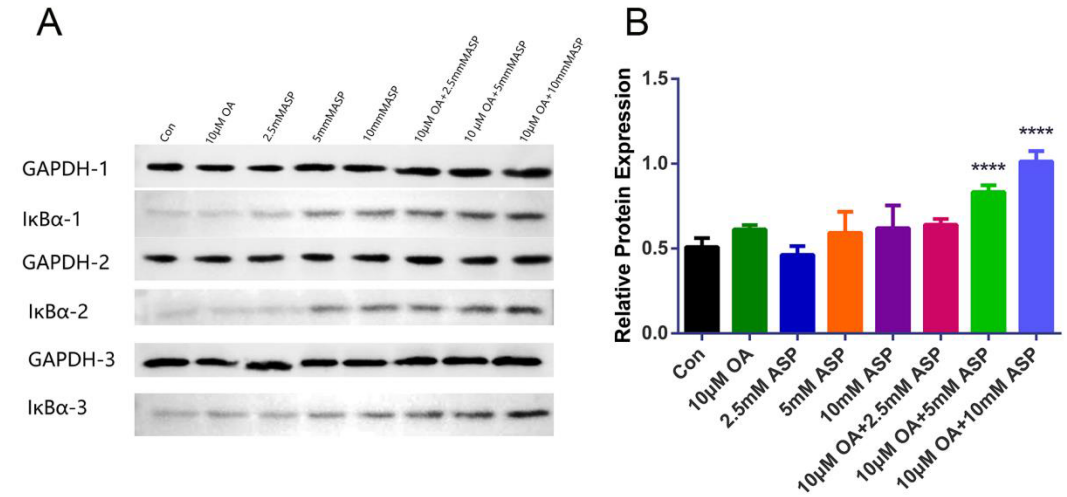

Figure 3. ***Western blot assay to detect the inhibition of IκBα expression in HT-29 cells by oleanolic acid enhanced aspirin.***
Western blots were performed in triplicate. Each bar represents the mean ± SD from three independent assays. **P < 0:01 ; *** P < 0:001..

degradation of IκBα induced by TNF-α by aspirin (Figure 4). This suggests that the combination treatment of oleanolic acid and aspirin enhanced the inhibition of PTGS2 expression by aspirin in colon cancer cells may be achieved by inhibiting the degradation of IκBα, which in turn inhibits the nuclear entry and transcriptional activity of NF-κB.

Discussion

Colorectal cancer, as one of the common malignant tumors of the gastrointestinal system, has a very high metastasis rate and mortality rate, and although various treatments have improved the therapeutic effect to a certain extent. It still cannot break through the problem of low survival rate and easy metastasis of CRC, and with the improvement of economic level in developing countries, living habits also tend to be more developed, which further increases the risk of CRC [21]. Most of the patients have progressed to intermediate and advanced stages by the time they are diagnosed. Failure of drug therapy and recurrence after surgery are also key factors affecting the prognosis of CRC patients [22].

Currently, natural products are receiving more and more attention in the field of anticancer therapy due to their good tolerability, lower toxicity, better therapeutic effect and improved quality of life of cancer patients. In recent years, the biological effects of triterpenoids such as hepatoprotective, analgesic, antitumor, anti-inflammatory and immunomodulatory are well known and applied in the prevention and treatment of various diseases. Triterpene saponins are broken down in the intestine, releasing triterpenoids that are absorbed and integrated into cell membranes to accomplish the regulation of signaling mechanisms that regulate various genes [23].

Oleanolic acid, is a pentacyclic triterpenoid widely present in plants in free or sugar-bound form. Oleanolic acid is a potent anti-tumor agent, and previous studies have investigated its anticancer potential in vitro and in vivo. However, its mechanism of anti-colorectal cancer remains incompletely elucidated. Clinical epidemiological studies have found that tumor mortality in colorectal cancer patients decreases exponentially with increasing cumulative aspirin intake [24]. However, aspirin is widely used as an over-the-counter common drug in clinical practice, and its associated adverse reactions are gradually increasing with its massive use, so it is important to pay close attention to its adverse reactions in clinical use. Aspirin inhibited the metastasis-related endpoints of colorectal cancer cells and also inhibited the migration and invasion of colorectal cancer cells [25]. Therefore, this study proposes the hypothesis of whether the combination of oleanolic acid and aspirin can enhance the anti-colorectal cancer effect of aspirin and provide a new direction for the development of new drugs for the treatment of colorectal cancer.

To verify this hypothesis, colorectal cancer cell lines HT-29 and HCT-116 cells were selected in this study, and four treatments were set: negative control group, oleanolic acid monotherapy group, aspirin (2.5, 5, 10 mM) monotherapy group, and combination group. The effects of oleanolic acid and aspirin on the proliferation of these 2 colorectal cancer cells were examined by MTT assay, and the results suggested that oleanolic acid and aspirin combination treatment enhanced the inhibitory effect of aspirin on the proliferation of colorectal cancer cells. In addition, the results of the assays at different time points showed that the inhibition of cell proliferation by oleanolic acid and aspirin was dependent on the length of treatment. This suggests that the combination treatment of oleanolic acid and aspirin has an inhibitory effect on the proliferation of colorectal cancer cells.

Aspirin is one of the most promising chemopreventive agents for CRC, and the main pathway for its beneficial effect on colorectal cancer is thought to be direct inhibition of COX-2/PTGS2. It has been reported that the benefits of using aspirin to reduce the risk of colorectal cancer are almost limited to COX-2-positive colorectal cancer [26]. Therefore, in this study, HT-29 cells were treated with different concentrations of oleanolic acid for 48 h. Oleanolic acid was found to dose-dependently inhibit PTGS2 expression in HT-29 cells. HT-29 cells were treated with the four treatments mentioned above, and the expression of PTGS2 in the cells was detected by western blot and RT-qPCR, and the combined drug group was found to significantly inhibit the expression of PTGS2 in the cells at the protein and mRNA levels.

IκB is a suppressor protein of nuclear factor (NF) -κB, which inhibits NF-κB activation at rest and activates NF-κB by phosphorylation of IκB protein when stimulating factors stimulate cells [27, 28]. IκB is an important member of the NF-κB signaling pathway and is involved in NF-κB activation and transcription [29]. Studies have shown that IκB expression deficiency may mediate the high expression of inflammatory factors in colorectal cancer, and targeting and regulating IκB has potential therapeutic promise [30].

Therefore, the present study examined the effects of the four treatments mentioned above on the expression of IκBα in HT-29 cells. The results showed that oleanolic acid enhanced the inhibition of IκBα expression by aspirin, suggesting that the enhanced inhibition of PTGS2 expression by aspirin in colon cancer cells by the combination of oleanolic acid and aspirin may be achieved by inhibiting the degradation of IκBα, which in turn inhibits the nuclear entry and transcriptional activity of NF-κB.

Conclusion

In conclusion, this study verified that the combination of oleanolic acid and aspirin could enhance the anti-colorectal cancer effect of aspirin through in vitro experiments, and the mechanism may be through the inhibition of the degradation of IκBα, which in turn inhibits the nucleation and transcriptional activity of NF-κB, thereby inhibiting the expression of COX-2 and thus the proliferation of colorectal cancer cells. This study suggests that our novel combination of oleanolic acid and aspirin may be a new therapeutic strategy for colorectal cancer, and more experiments are needed to validate the combination in the future.

Data sharing statement

No additional data are available.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was funded by the Natural Sciences Foundation of Fujian Province (2021J011393) and the Natural Sciences Foundation of Fujian Province (2019J01592).

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