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Apigenin combined with cisplatin suppressed the progression of colorectal cancer by targeting the KRT23/Wnt/β-catenin signaling pathway

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Abstract

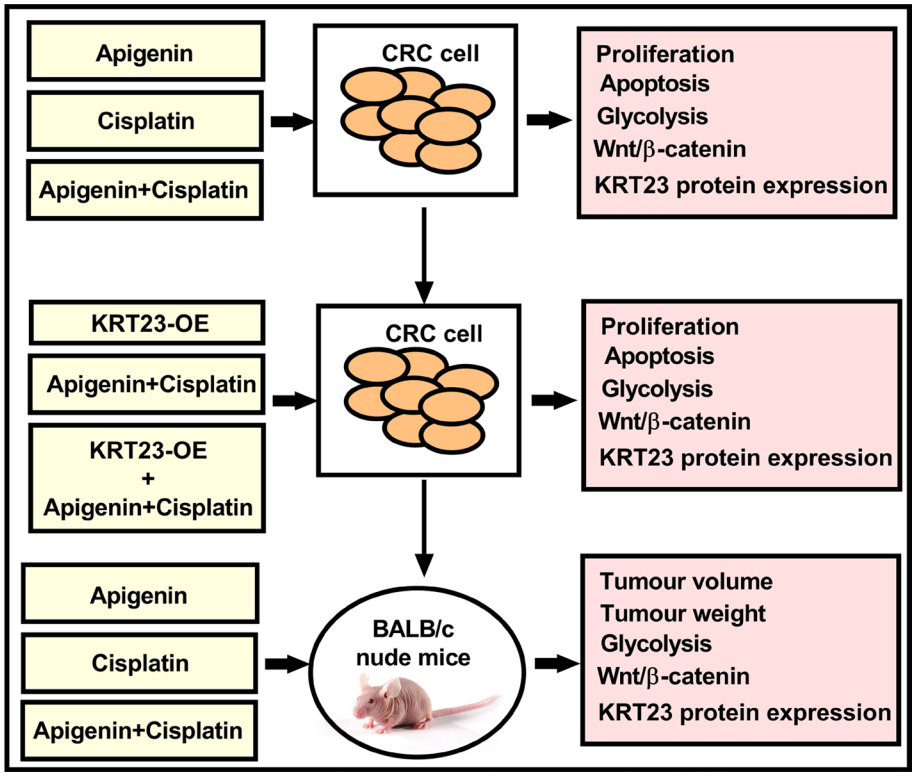
Colorectal cancer (CRC) represented a pervasive manifestation of malignant neoplasia within the digestive tract. Apigenin, exhibiting a multitude of physiological attributes and pharmacological actions, has undergone extensive scrutiny for its antitumor efficacy and benign toxicity profile. Cisplatin (DDP)-centered chemotherapy constituted a pivotal aspect of multidisciplinary therapeutic strategies. Nevertheless, resistance to DDP posed a considerable impediment to the efficacy of CRC chemotherapy. The aim of this investigation was to assess the impact of combining Apigenin with DDP on the proliferation and apoptotic processes of human CRC cells, while also delving into the underlying mechanisms. HCT116 and SW480 were cultivated and subjected to treatment with Apigenin (API) either as a monotherapy or in combination with cisplatin (DDP). Cell viability, proliferation, cycle distribution, apoptosis, migration, invasion and inflammatory factors were assessed. Western blot analysis was performed to detect the protein expression levels of Glut1, HK-2, KRT23, and β-catenin. In comparison to other treatment groups, the combined API and DDP group exhibited a more potent suppressive effect on cellular proliferation, migration, invasion, and glycolysis, while also enhancing apoptotic activity. Additionally, the combined API and DDP treatment group led to a reduction in the expression levels of KRT23, β-catenin, HK-2, and Glut1. Intriguingly, this combined treatment group demonstrated significantly elevated levels of TNF-α, IL-6, and IL-8 compared to the other groups. Notably, the overexpression of KRT23 was capable of reversing the changes induced by the combined API and DDP treatment. In vivo studies further validated that the combined API and DDP treatment suppressed tumor growth by inhibiting the expression of KRT23 and β-catenin. The present findings indicated that the combination of API with DDP has the potential to enhance colorectal cancer therapy through the modulation of the KRT23/Wnt/β-catenin signaling pathway. Our research may offer fresh perspectives and novel molecular therapeutic strategies for the treatment of colorectal cancer.

Highlights

1.The effect of API combined with DDP on colorectal cancer cells were studied for the first time.
2.API combined with DDP can decrease the expression of KRT23 and Wnt/β-catenin signaling pathway.
3.The regulatory relationship between KRT23 and Wnt/β-catenin signaling pathway had been reported for the first time.
4.API combined with DDP regulated the CRC cell glycolysis and Inflammatory factors.

Keywords Apigenin (API), Cisplatin (DDP), Colorectal cancer (CRC), KRT23, Wnt/β-catenin signaling pathway

Graphical Abstract



1 Introduction

Colorectal cancer (CRC) stands as one of the most prevalent and lethal malignancies globally, distinguished by its origin in the glandular epithelium of the colon and rectum, thereby posing a formidable challenge to human health [1]. The International Agency for Research on Cancer (IARC) projects a staggering 63% increase in new cancer cases by 2040, culminating in 3.2 million annual diagnoses. While the therapeutic landscape for CRC encompasses standard systemic chemotherapy, alternative therapies targeting oncogenic progression and metastatic mechanisms, immunotherapy, and combination therapies, their efficacy remains suboptimal, contributing to a high mortality rate annually [2]. Notably, despite chemotherapy's demonstrated capacity to alleviate tumor burden and extend survival, it is primarily regarded as a palliative measure, as the majority of CRC patients ultimately develop drug resistance [3]. Despite recent technological advancements and intricate research endeavors, CRC's survival rate remains dismally

low, largely attributed to late diagnosis. Hence, unraveling the mechanisms underlying CRC development is imperative for facilitating early diagnosis and devising more potent therapeutic strategies. Furthermore, elucidating the signaling pathways implicated in CRC progression and metastasis, particularly the Wnt/β-catenin signaling pathway, holds promise for the development of novel CRC therapeutics.

Flavonoids constitute a class of bioactive plant-derived compounds exhibiting remarkable properties, including antioxidant, anti-inflammatory, antibacterial, antifungal, and antitumor activities. These attributes render them highly significant in the realms of oncology and pharmacology [4]. Apigenin (4',5,7-trihydroxyflavone), a specific flavonoid abundant in fruits and vegetables, possesses diverse biological activities, notably its anticancer properties [5]. In recent years, extensive research has focused on the role of apigenin in tumorigenesis. Studies have demonstrated that apigenin can effectively inhibit CRC in both in vitro and in vivo models by curbing glycolysis in LS-174 T and HCT-8 cells [6]. Notably, apigenin achieves significant tumor growth suppression without eliciting adverse reactions. These observations hint at the potential of apigenin as a promising therapeutic agent for CRC.

Cisplatin (DDP) stands as one of the most ubiquitously employed anticancer agents globally, primarily utilized in the management of colorectal, hepatic, and lung carcinomas [7]. However, a pivotal challenge faced by cancer patients undergoing DDP therapy is the emergence of numerous resistance mechanisms during treatment, ultimately culminating in suboptimal therapeutic outcomes and a constellation of adverse effects. Especially in colorectal cancer patients, the therapeutic effect of DDP was still limited by intrinsic drug resistance. Several mechanisms of DDP resistance have been elucidated, such as insufficient DNA binding, reduced virulence, promotion of DNA repair, dysregulation of transporter protein expression, and alterations in gene expression and activation. Research endeavors have suggested that plant extracts or specific monomeric components of traditional Chinese medicine (TCM) have the capacity to mitigate the side effects associated with DDP therapy and potentiate its therapeutic efficacy [8, 9].

Keratin, a structural protein abundant in epithelial cells, plays a pivotal role in membrane trafficking, signaling cascades, protein synthesis, and cellular motility [10]. A wealth of research has elucidated its involvement in diverse physiological processes associated with cancer, encompassing proliferation, apoptosis, migration, and invasion [11]. Among the keratin isoforms, Keratin 23 (KRT23), belonging to the acidic type I keratin family, has recently garnered attention. In a recent investigation by Li et al., the deletion of KRT23 was found to impact the proliferative and apoptotic cycles of gastric cancer cells [12]. Other studies have found that keratin 23 (KRT23) is strongly expressed in colon adenocarcinomas but absent in normal colon mucosa [13]. KRT23 is correlated with cell proliferation and migration through regulating the ERK1/2 and p38 signaling pathways [12]. In addition, some studies have suggested that KRT23 may serve as a target for the clinical treatment of colorectal cancer [14]. Therefore, KRT23 was selected as a research target. This study furnishes compelling evidence for the role of KRT23 in the onset and progression of cancer.

The Wnt/β-catenin signaling pathway, alternately referred to as the canonical Wnt signaling pathway, is a well-established regulatory mechanism that plays a pivotal role in numerous physiological processes within tumor cells, encompassing proliferation, apoptosis, migration, invasion, cell cycling, and angiogenesis [15]. β-catenin, a

functional effector within this signaling cascade, undergoes modifications and degradation, which are critical events in the oncogenesis of various cancers [16]. In the context of colorectal cancer (CRC) pathogenesis, the Wnt/β-catenin signaling pathway is widely acknowledged as a key driver of colon cancer progression and stands as one of the most representative signaling mechanisms involved [17]. Therefore, although tumor occurrence and development involve numerous signaling pathways, we selected the Wnt/β-catenin signaling pathway for further investigation.

When designing this study, we considered that cisplatin treatment is prone to causing drug resistance in patients, while the traditional Chinese medicine monomer apigenin is relatively safe and has no toxic side effects. Therefore, we hypothesize that the combination of these two treatments for colorectal cancer may have better therapeutic effects. In this investigation, our primary objective was to scrutinize the effects of apigenin (API), in conjunction with cisplatin (DDP), on the proliferation, apoptosis, migration, invasion, and inflammatory responses of colorectal cancer (CRC) cells. Furthermore, we sought to delve into the underlying molecular mechanisms governing these phenomena. Our results revealed that the combined administration of apigenin and cisplatin exhibited potent regulatory effects on CRC progression, primarily through modulation of the KRT23/Wnt/β-catenin signaling axis.

2 Material and methods

2.1 Cell culture

The CRC cell lines, HCT116 and SW480, were acquired from BeNa Biotechnology Co. Ltd. (catalog numbers BNCC287750 and BNCC100604, respectively, located in Xinyang, China). HCT116 cells were maintained in RPMI 1640 medium (Solarbio, catalog number 31800, Beijing, China), supplemented with 10% fetal bovine serum (Gibco, catalog number 16000-044, MD, USA), and incubated at 37 °C in an atmosphere containing 5% CO_2. SW480 cells were cultivated in Leibovitz's L-15 Cell Culture Medium (Solarbio, catalog number LA9510, Beijing, China), also supplemented with 10% fetal bovine serum, and maintained at 37 °C. Both media were further enriched with penicillin/streptomycin at a concentration of 100 U/ml. Experimental procedures were initiated once the cells reached 80% confluence.

2.2 Cell transfection of keratin 23 (KRT23) gene overexpression vector

The pcDNA3.1-KRT23 plasmid for overexpressing the KRT23 gene was sourced from Wuhan Aoke Dingsheng Biotechnology Co., Ltd. By the way, KRT23 construct was untagged. This plasmid was subsequently transfected into HCT116 and SW480 cells utilizing the QuickShuttle-Enhanced transfection reagent (catalog number KX0110042, Biodragon, Suzhou, China). The transfection protocol adhered strictly to the manufacturer's guidelines.

2.3 Assessment of cell viability with cell counting kit-8(CCK8)

3×10^3 CRC cells were plated in a 96-well plate and incubated with apigenin (API) (Solarbio, catalog number IA0400) or cisplatin (DDP) (Solarbio, catalog number D8810) for durations of 24 and 48 h. Following incubation, the culture medium was aspirated, and 100 μl of serum-free medium containing 10% CCK-8 (catalog number 40203ES60,

Yeasen, Shanghai, China) was added to each well for 1–4 h. The absorbance at a wavelength of 450 nm was then quantified using a spectrophotometric analyzer.

2.4 Flow cytometric analysis

Cell apoptosis was assessed via flow cytometry. Cells were harvested using trypsin devoid of EDTA (Solarbio, catalog number T1350), and then incubated according to the meticulous instructions provided with the Annexin V-FITC/PI Apoptosis Detection Kit (catalog number 40302ES20, Yeasen, Shanghai, China). Subsequently, the proportion of apoptotic cells was determined using a flow cytometer.

HCT116 and SW480 cells were collected and fixed in 70% ethanol at 4 °C for an overnight period. Subsequently, the fixed cells were stained with RNase A and propidium iodide (PI; catalog number 40301ES60, Yeasen, Shanghai, China) at room temperature, protected from light, for a duration of 30 min. The stained cells were then evaluated using flow cytometry. Ultimately, the distribution of cells across different phases of the cell cycle was analyzed utilizing FlowJo software.

2.5 Cell invasion assay

The invasive capacity of the cells was evaluated using the Transwell assay. The 24-well Transwell chambers, equipped with 8 μm polycarbonate membranes, were pre-coated with a 500 ng/mL matrix adhesive solution and allowed to solidify. Subsequently, 10% fetal bovine serum (FBS) medium was added to the lower chamber, while serum-free medium was placed in the upper chamber. The cells were then seeded into the upper chamber. After an incubation period of 24 h, the cells adhering to the upper surface of the membrane were delicately removed. Following this, the cells were fixed with methanol and stained with 0.5% crystal violet. Ultimately, five random fields of view were selected, and the cells were photographed and enumerated.

2.6 Wound-healing assay

Approximately 3×10^5 cells were plated onto a six-well dish until they formed a confluent monolayer. A scratch was introduced across the cell layer using the tip of a pipette, followed by three gentle washes with phosphate-buffered saline (PBS). The cells were then cultured in either RPMI-1640 or L-15 medium supplemented with 1% fetal bovine serum (FBS). Cell images were captured at 0 and 24 h post-scratching. Ultimately, the width of the scratch gap at these time points was quantified.

2.7 Cell colony formation assay

For the colony formation assay, approximately 2000 cells were plated in a six-well dish and allowed to grow. After a period of 14 days, the cells were fixed with paraformaldehyde for 15 min and subsequently stained with 0.1% crystal violet for 15 min. Following thorough washing with phosphate-buffered saline (PBS), the colonies were imaged and enumerated.

2.8 Enzyme linked immunosorbent assay (ELISA) for inflammatory cytokines detection

The cell supernatant was harvested for the quantification of inflammatory cytokines (TNF-α, IL-6, and IL-8) using the enzyme-linked immunosorbent assay (ELISA) method. Each sample was processed in strict accordance with the manufacturer's instructions

provided with the respective ELISA kits (MEIMIAN, catalog numbers MM-0122H1, MM-0049H1 and MM-1558H1, Yancheng, China), and the experiments were conducted in triplicate to ensure reproducibility.

2.9 Subcutaneous xenograft model

For the establishment of the in vivo xenograft tumor mouse model, 24 male BALB/c nude mice aged 4–5 weeks were sourced from Liaoning Changsheng Biotechnology Co., Ltd. and housed in a specific pathogen-free (SPF) facility. The mice were injected subcutaneously with a single-cell suspension, and the number of cells inoculated was 5×10^7. Upon the emergence of palpable tumors (reaching a volume of approximately 100 mm^3), the mice were randomized into four groups, with six mice per group. The control group received intraperitoneal (i.p.) injections of saline (vehicle), while the treatment groups were administered apigenin (API; 25 mg/kg), cisplatin (DDP; 3 mg/kg), or a combination of both, every other day. Tumor volumes (mm^3) were measured bi-daily and calculated using the formula: $(\text{length} \times \text{width}^2)/2$. The maximum tumor volume must not exceed 1000 mm^3 to ensure the welfare of experimental animals as well as the safety and scientific nature of the research. During the experiment, we strictly observed and recorded the growth of tumors in nude mice. We confirmed that the maximal tumor size/burden was not exceeded the rules of Ethics Committee. After 14 days of treatment, the mice were euthanized via excess CO_2 asphyxiation. Once the animals became unresponsive with dilated pupils, the CO_2 flow was terminated, and a further 1–3 min of observation ensured their demise. The tumor tissues were then excised, weighed, and subjected to further analysis. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou Anorectal Hospital, adhering to ethical guidelines for animal research.

2.10 RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cells using Trizol® total RNA isolation reagent (TaKaRa, catalog numbers 9109, Dalian, China) according to the manufacturer's protocol. Then, cDNA was synthesized and Real-time PCR was performed. The relative expression levels of KRT23 were calculated by the $2^{-\Delta\Delta Ct}$ method [18].

2.11 Western blot analysis

Cellular proteins were extracted from HCT116 and SW480 cells using Radio-Immunoprecipitation Assay (RIPA) buffer (purchased from KeyGen, China) supplemented with 1% phenylmethanesulfonyl fluoride (PMSF). The protein concentration was quantified using a BCA Protein Assay Kit sourced from Solarbio (Beijing, China). Subsequently, 30 μg of total protein were resolved by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk for 2 h at room temperature, followed by overnight incubation at 4 °C with a primary antibody targeting β-actin (Biodragon, catalog numbers B1029, Suzhou, China), β-catenin (Biodragon, catalog numbers BD-PM3403), Glut1 (proteintech, catalog numbers 66290–1-Ig, Wuhan, China), HK-2 (proteintech, catalog numbers 66974–1-Ig), and KRT23 (Biodragon, catalog numbers BD-PN0242), each diluted to 1:1,000. After washing the membranes three times with TBST for 10 min each, they were incubated

with the respective secondary antibodies (Biodragon, catalog numbers BF03001 and BF03008) for 1 h. Subsequently, the membranes were subjected to enhanced chemiluminescence (ECL) substrate detection. Finally, the protein bands were quantified using an image analyzer.

2.12 Statistical analysis

The data were presented as the mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software (version 6.0, San Diego, CA). Comparisons between two distinct groups were performed using the independent-samples t-test, whereas comparisons involving multiple factors were analyzed through analysis of variance (ANOVA). A P-value < 0.05 was deemed statistically significant.

3 Results

3.1 API and DDP inhibited CRC cell proliferation

Following a 24/48 h exposure to varying concentrations of API or DDP, the viability of HCT116 and SW480 colorectal cancer (CRC) cells was evaluated using the CCK8 assay, thereby confirming the inhibitory potency of API and DDP on CRC cell line proliferation. As shown in Fig. 1, a certain degree of high concentration of apigenin or cisplatin can inhibit cell viability, while the effect is not very significant at lower concentrations. Based on those, researchers also referred to relevant literature and believed that whether it was 24 h or 48 h, for both cells, treatment with 40 μM API and 20 μM DDP resulted in significant inhibition of cell viability, and both were close to the IC50 range. Upon conducting a thorough analysis, the researchers selected concentrations of 40 μM API and 20 μM DDP for subsequent experimental procedures. These findings suggest that both API and DDP effectively suppress CRC cell proliferation.

3.2 API combined with DDP showed more efficiency in suppressing CRC proliferation, migration, invasion, and promoting CRC cell apoptosis

To assess the effects of API alone or in combination with DDP on colorectal cancer, HCT116 and SW480 cells were treated with these agents. CCK-8 assays revealed that the combination of API and DDP exhibited greater inhibitory effect on cellular proliferation than either agent alone (Fig. 2A, B). Clonogenic assays further demonstrated that the combination significantly inhibited cell proliferation (Fig. 2C). To explore whether the anti-proliferative effects of API and DDP were due to cell cycle arrest, flow cytometry was employed to analyze the cell cycle phase distributions of CRC cells treated with API alone, DDP alone, or their combination. Both API and DDP induced G0/G1 phase arrest compared to the control group, with the combination being more effective (Fig. 2D). Additionally, the apoptotic rate was significantly increased in the combination group (Fig. 2E). Next, the migratory and invasive capabilities of CRC cells were evaluated. Transwell assays and wound-healing experiments indicated that, compared to other groups, the combined treatment group exhibited a stronger inhibitory effect on migration and invasion (Figure S1A and S1B). Collectively, these findings demonstrate that the combination of API and DDP is more effective in suppressing CRC proliferation, migration, invasion, and promoting CRC cell apoptosis.

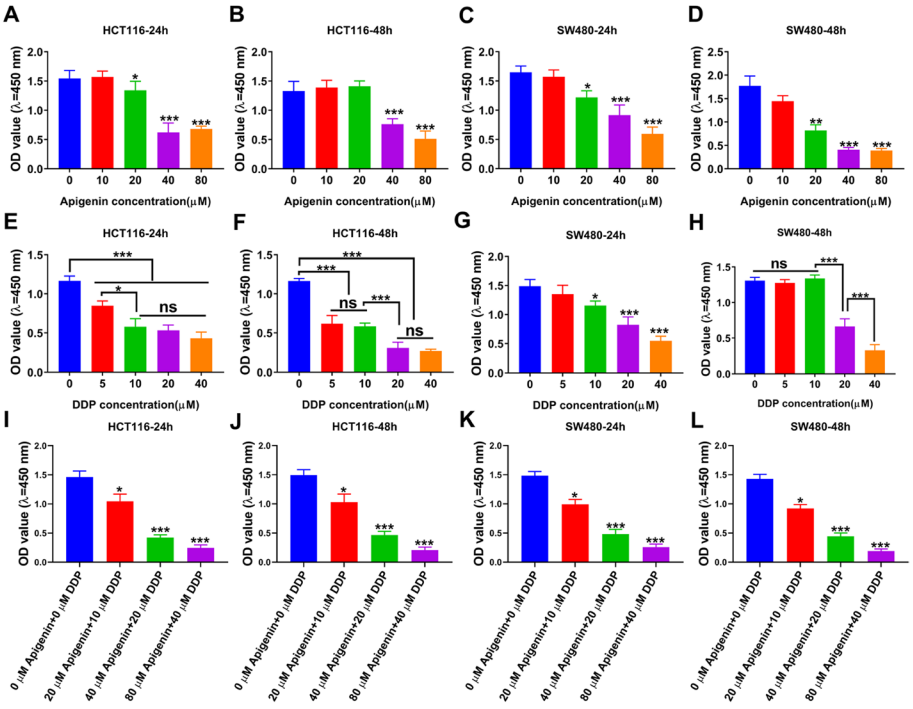


Fig.1 Effects of API and DDP on the CRC cell proliferation. **A** HCT116 cell proliferation viability after treatment with API for 24 h was detected by CCK8 assay. **B** HCT116 cell proliferation viability after treatment with API for 48 h was detected using CCK8 assay. **C** SW480 cell proliferation viability after treatment with API for 24 h was detected by CCK8 assay. **D** SW480 cell proliferation viability after treatment with API for 48 h was detected by CCK8 assay. **E** HCT116 cell proliferation viability after treatment with DDP for 24 h was detected by CCK8 assay. **F** HCT116 cell proliferation viability after treatment with DDP for 48 h was detected by CCK8 assay. **G** SW480 cell proliferation viability after treatment with DDP for 24 h was detected by CCK8 assay. **H** SW480 cell proliferation viability after treatment with DDP for 48 h was detected by CCK8 assay. **I** HCT116 cell proliferation viability after treatment with API and DDP for 24 h was detected by CCK8 assay. **J** HCT116 cell proliferation viability after treatment with API and DDP for 48 h was detected by CCK8 assay. **K** SW480 cell proliferation viability after treatment with API and DDP for 24 h was detected by CCK8 assay. L: SW480 cell proliferation viability after treatment with API and DDP for 48 h was detected by CCK8 assay. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

3.3 Glut1, HK-2, Wnt/β-catenin signaling pathway, KRT23, and inflammatory cytokines were involved in regulation of API combined with DDP on colorectal cancer cells

Given that prior studies have established the involvement of the Wnt/β-catenin pathway and glycolysis pathway in the initiation and progression of CRC, we concurrently investigated the expression changes of β-catenin protein, a pivotal component of the Wnt/β-catenin pathway, and Glut1, HK-2, two key protein of glycolysis pathway in our experiment. As illustrated in Fig. 3A, B, the expression level of Glut1, HK-2 and β-catenin protein was reduced in both the API-alone, DDP-alone and API-combined-with-DDP groups compared to the control group, with the most significant decrease observed in the API-combined-with-DDP group. Having elucidated the inhibitory impact of API alone, DDP alone, or API conjunction with DDP on colorectal cancer cells, we utilized the western blotting method to quantify the expression levels of the KRT23 protein. Through western blotting analysis, we observed that the relative expression of KRT23 protein was markedly decreased in both the API-alone, DDP-alone and API-combined-with-DDP groups compared to the control group (Fig. 3C). These findings suggest that KRT23, Glut1, HK-2 and the Wnt/β-catenin pathway are implicated in the mechanisms by which API and DDP exert their effects on CRC cells. Furthermore,

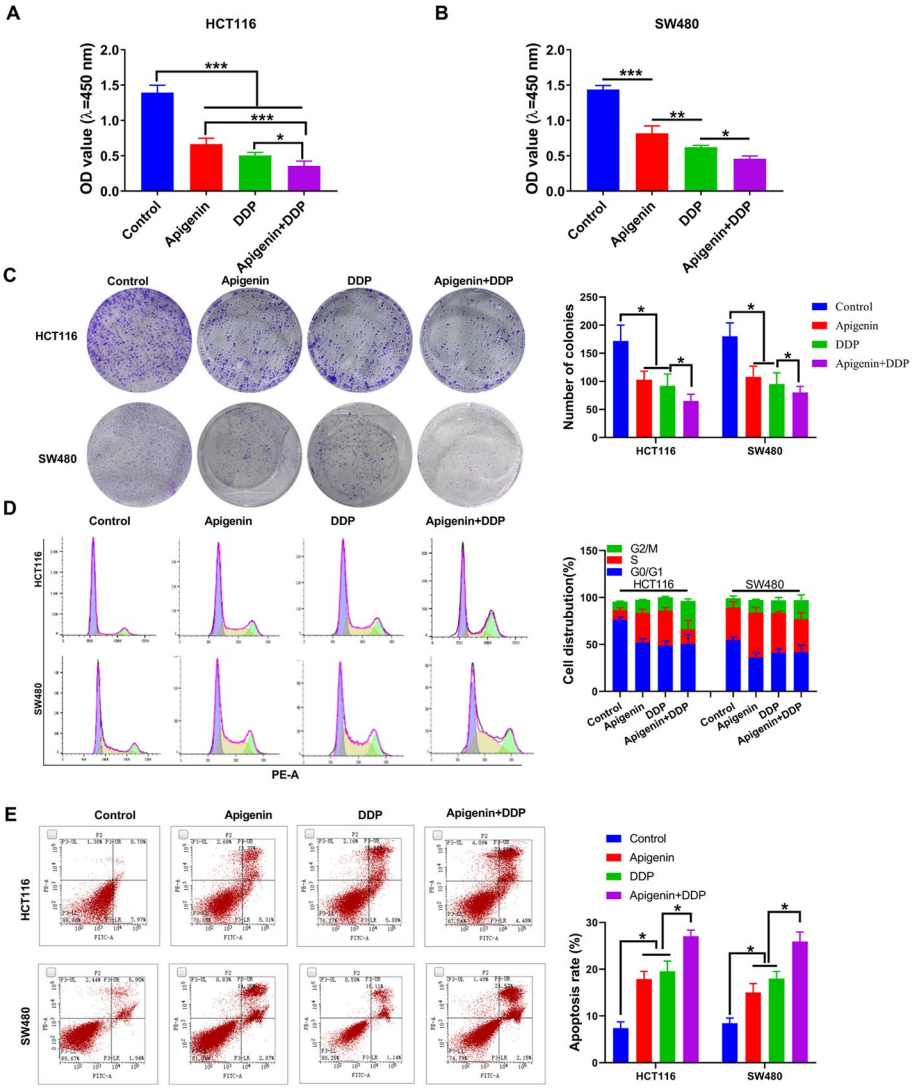


Fig. 2 API combined with DDP to inhibit CRC cell proliferation, migration, invasion and promote CRC cell apoptosis. **A**, **B**CCK-8 assays were performed to determine the proliferation ability of CRC cells after treatment with API combined with DDP. **C** Colony formation assays were performed to measure the proliferation ability of CRC cells. **D** Cell cycle distribution (G0/G1(lower), S(middle), and G2/M phases(upper)) in CRC cells was analyzed using Flow Cytometry. **E** Flow cytometric analysis was performed to determine the apoptosis of CRC cell apoptosis by Annexin V-FITC/PI double staining**.** API(40 μM), DDP(20 μM). Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

the study also assayed the levels of common inflammatory cytokines (TNF-α, IL-6 and IL-8) and found that the expression levels of inflammatory cytokines decreased in the apigenin-treated group, while those in the cisplatin-treated group increased. The combined apigenin and cisplatin group exhibited levels close to normal (Fig. 3D–F). These results reveal that glycolysis pathway, Wnt/β-catenin signaling pathway, KRT23 protein, and inflammatory cytokines were involved in regulation of API combined with DDP on colorectal cancer cells, but further research is needed to elucidate the specific underlying mechanisms.

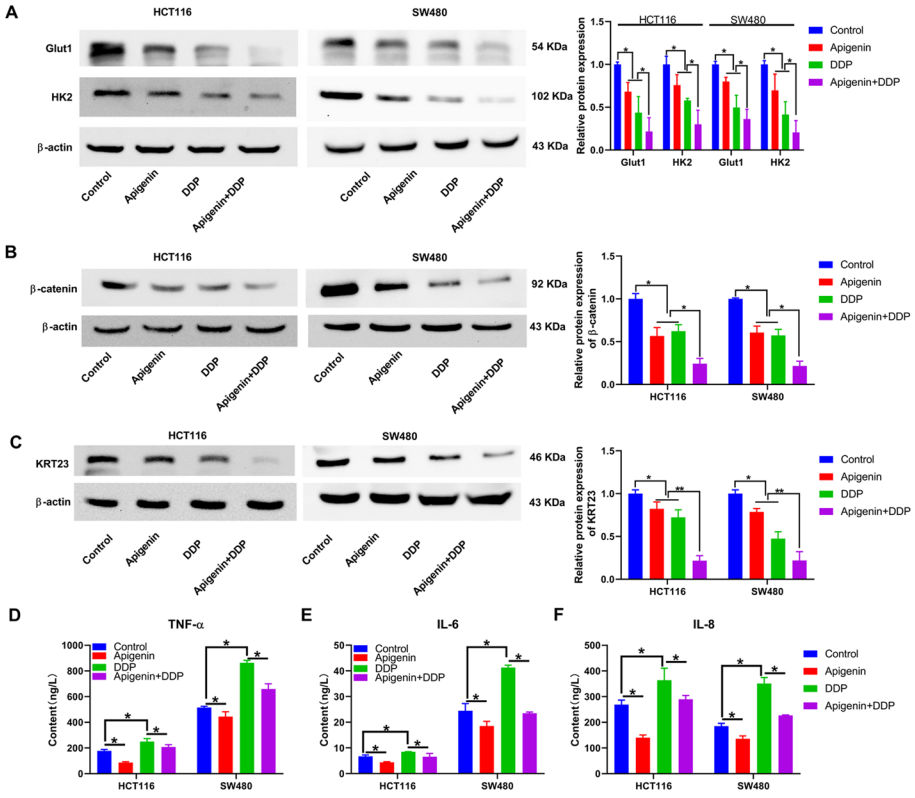


Fig. 3 Glycolysis, Wnt/β-catenin signaling pathway, KRT23 and inflammation were associated with regulation of API combined with DDP on colorectal cancer cells. **A**The relative expression of KRT23 were detected in CRC cells using Western blotting. **B** Protein expression of β-catenin in CRC cells was determined by Western blotting. β-actin was used as a loading control. **C** The protein level of KRT23 in CRC cells was detected by Western blot. **D**–**F** The content of TNF-α, IL-6 and IL-8 was assessed by ELISA assay. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

3.4 KRT23 regulates the function of API combined with DDP on the cell viability, apoptosis, migration, and invasion colorectal cancer cells

To delve deeper into the relationship between KRT23 and the drug combination of API with DDP, as well as the role of KRT23 in CRC cell proliferation, we initially constructed a KRT23 overexpression vector and successfully transfected it into CRC cells using the QuickShuttle-Enhanced transfection reagent. Subsequently, real-time PCR was conducted to verify the overexpression of KRT23 in these cells (Fig. 4A, B). Furthermore, we evaluated cell viability, migration, and invasion abilities to ascertain whether KRT23 plays a role in CRC cells treated with the API-DDP combination. As depicted in Fig. 4C–G, and Figure S2A and S2B, the combination of the drug (API with DDP) and KRT23 overexpression exhibited a potent recovered effect on CRC cell viability, cell cycle, migration, and invasion, surpassing that observed in the drug-only, KRT23-only, and control groups. Especially for the cell cycle, the drug (API with DDP) treatment can cause cycle arrest, KRT23-OE group can promote cell cycle, and API + DDP + KRT23-OE group treatment can make the cell cycle approach the normal cycle state of tumor cells. Notably, Fig. 4C illustrates that the drug combination paired with KRT23 overexpression significantly restored the apoptosis rate compared to the other groups.

Collectively, these findings indicate that the combination of the drug (API with DDP) and KRT23 overexpression can reverse the inhibitory effects of the drug combination

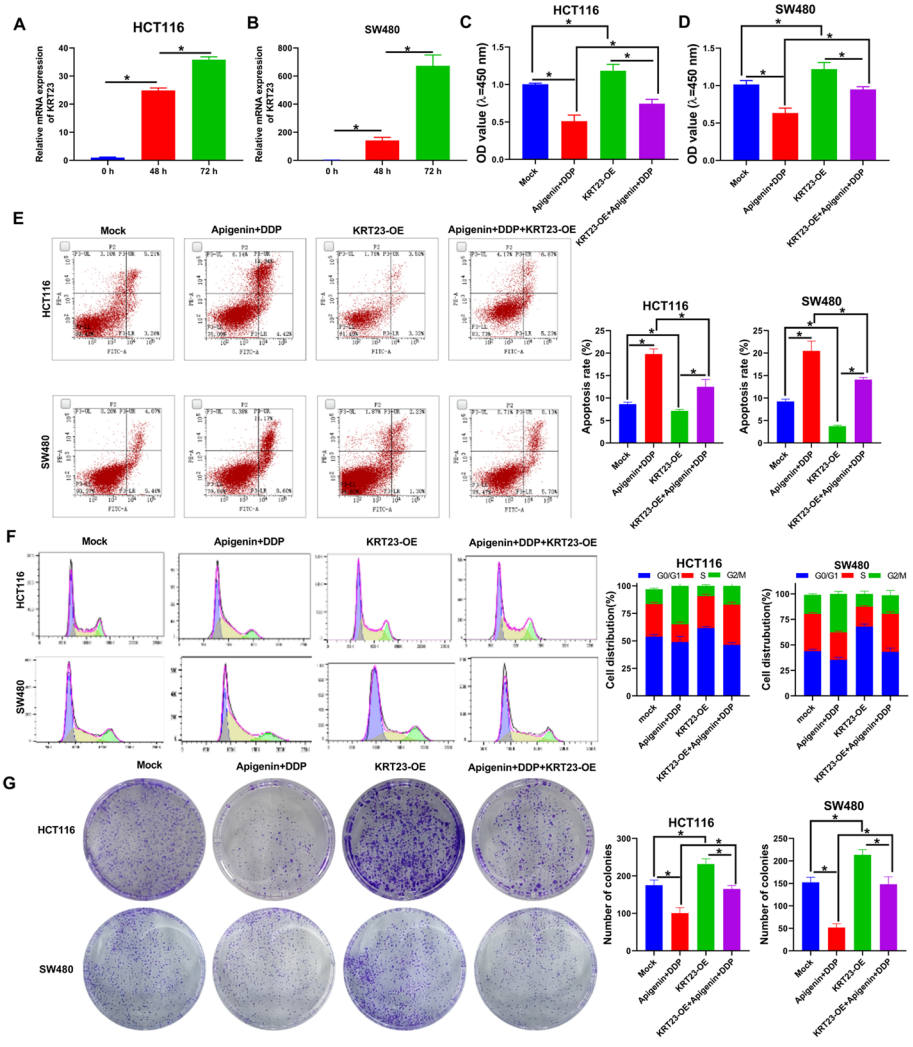


Fig. 4 Overexpression of KRT23 gene could restore the inhibition of API combined with DDP on colorectal cancer cells. **A**, **B** Transfection efficiency of KRT23-OE was determined by qPCR assay. **C**, **D** The viability of CRC cells was evaluated by CCK-8 assay. **E** The apoptosis of CRC cells was assessed by flow cytometry assay. **F** Flow cytometry was used to detect the CRC cell cycle. **G** The proliferation of CRC cells was detected by clone formation assay. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

on colorectal cancer cells, suggesting a complex interplay between KRT23 and the drug (API and DDP) regimen in CRC cell biology.

3.5 The effect of Apigenin combined with DDP on Glut1, HK-2, and Wnt/β-catenin signaling pathway could be regulated by KRT23 protein

Subsequently, we conducted a Western blot analysis to examine the expression levels of the Glut1, HK-2, KRT23 and β-catenin protein. As anticipated, the results demonstrated that KRT23 overexpression significantly potentiated Glut1, HK-2, and β-catenin expression in comparison to the sole treatment with the drug combination (API combined with DDP) (Fig. 5A, B). In aggregate, the aforementioned findings suggest that the Glut1, HK-2, and Wnt/β-catenin signaling pathway may be modulated by KRT23. In addition, when KRT23-OE was used in combination with drugs (Apigenin + DDP), the protein expression level returns to a state close to normal level. Collectively, these

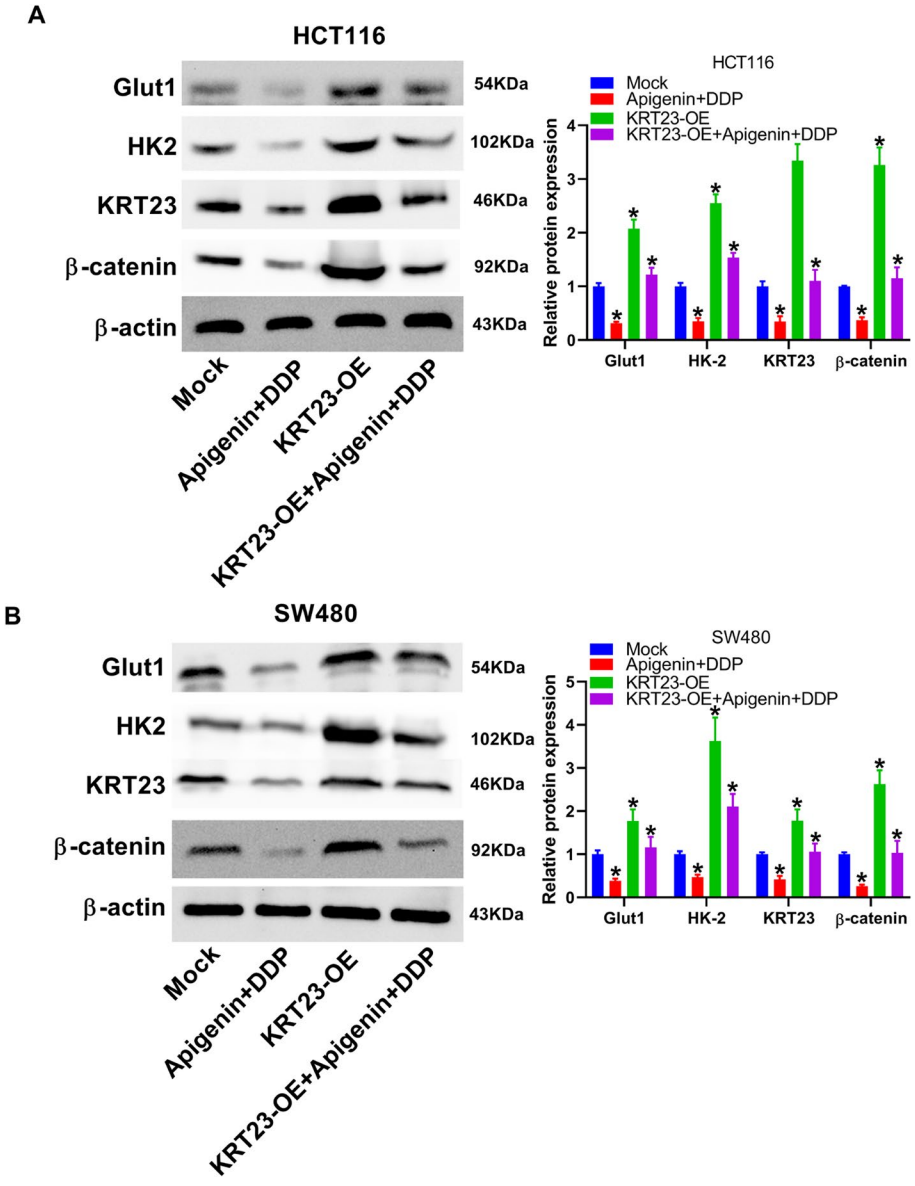


Fig. 5 Overexpression of KRT23 gene could cause β-catenin, Glut1, HK-2 and KRT23 protein changes in the CRC cells. **A** The protein expression of β-catenin, Glut1, HK-2 and KRT23 in HCT116 cells was detected by Western blotting assay. **B** The protein expression of β-catenin, Glut1, HK-2 and KRT23 in SW480 cells was detected by Western blotting assay. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

findings indicate that the effect of Apigenin combined with DDP on Glut1, HK-2, and Wnt/β-catenin signaling pathway could be regulated by KRT23 protein.

3.6 API combined with DDP suppressed CRC tumour progression in vivo

To ascertain whether API could augment the therapeutic efficacy of DDP in vivo, we utilized male BALB/c nude mice (aged 5–6 weeks) as our animal model. These mice were subcutaneously inoculated with 5×10^7 HCT116 cells. Seven days post-inoculation, the mice were stratified into four groups (n = 6) based on tumor volume. The mice in these groups were administered either PBS as a control, API (25 mg/kg/day), DDP (3 mg/kg), or a combination of DDP and API. After 14 days of treatment, the mice were euthanized,

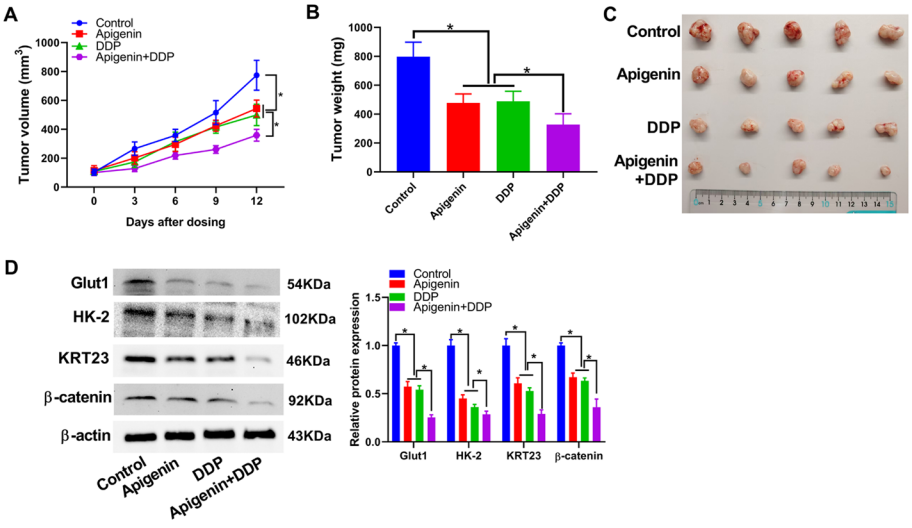


Fig. 6 API combined with DDP inhibited CRC tumour growth in vivo.**A**, **B** The tumor volumes and tumour weight were measured every 3 days after the injection. **C** Photographs illustrating xenografted tumors. **D** Western blot analysis was performed to measure the expression level of β-catenin, Glut1, HK-2 and KRT23 in tumor tissues. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups

and their tumor tissues were harvested. The results revealed that the API + DDP combination therapy led to a further reduction in both tumor volume and tumor weight compared to monotherapy with either API or DDP (Fig. 6A–C). Additionally, we conducted Western blot analysis to assess the expression levels of β-catenin, Glut1, HK-2, and KRT23. The results demonstrated that the expression levels of these proteins were decreased in the API + DDP group compared to the monotherapy groups (Fig. 6D). Collectively, these findings indicated that API combined with DDP suppressed the CRC tumor growth progression by targeting the KRT23/Wnt/β-catenin signaling pathway and regulating glycolysis pathway in vivo.

4 Discussion

In this investigation, we initially examined the impact of apigenin and cisplatin on cellular viability, subsequently delving into an analysis of the effects of their combination on cellular proliferation, apoptosis, migration, and invasion. Our findings revealed that the conjunction of apigenin with cisplatin exhibits a superior capacity to suppress cellular proliferation, migration and invasion, while concurrently augmenting cellular apoptosis. Thereafter, we embarked on an exploration of the molecular regulatory mechanisms underlying the modulation of colorectal cancer progression by the apigenin-cisplatin combination. Our research unveiled the involvement of the KRT23 gene, the glycolysis pathway, the Wnt/β-catenin signaling pathway, and inflammatory cytokines in this regulatory network. These explorations have never appeared in other studies, which highlights the novelty and depth of this study. To further substantiate our observations, we conducted experiments in which KRT23 was overexpressed within cells, confirming that it can partially reinstate the inhibitory efficacy of the drugs (API and DDP) on colorectal cancer. Additional studies have demonstrated that the expression of the glycolysis pathway and Wnt/β-catenin signaling pathway is also responsive to the inhibitory impact of KRT23-restored drugs on colorectal cancer. Ultimately, we conducted in vivo

experiments to conclusively validate the inhibitory action of the apigenin-cisplatin combination on colorectal cancer.

In the year 2024, Shao and colleagues conducted a groundbreaking study on the active constituents of the traditional Chinese medicine purslane, revealing that apigenin, a potent component within this herbal remedy, holds promise for the prevention and treatment of colitis and associated colorectal cancer [19]. Their further research elucidated that apigenin facilitates intestinal mucosal barrier repair and exerts anti-cancerous effects by inhibiting the STAT3-NF-κB signaling cascade. In the realm of liver cancer research, Chou and team discovered that apigenin exerts its inhibitory influence on cellular growth and induces cell cycle arrest via the MED28-mediated AKT/mTOR signaling pathway [20]. A multitude of studies have highlighted the multifaceted anti-cancer mechanisms of apigenin, which include the induction of cell cycle arrest and apoptosis, as well as the suppression of migration, invasion, and angiogenesis [21]. Zhang and colleagues further contributed to this body of knowledge by demonstrating that apigenin inhibits the proliferation, migration, and invasion of 4T1 cells, while promoting cellular apoptosis by modulating the caspase-3/cleaved-caspase-3 and Bcl-2/Bax ratios [22]. Additionally, apigenin exhibits anti-cancerous properties through the inhibition of the PI3K/AKT/Nrf2 signaling pathway. As research into the anti-cancerous effects of apigenin deepens, it has become increasingly evident that this compound is intricately linked to numerous signaling pathways, including the PI3K/AKT, MAPK/ERK, JAK/STAT, NF-κB, and Wnt/β-catenin pathways [23].

Keratin 23 (KRT23), a novel addition to the KRT gene family, exhibits heightened expression in specific tumor tissues and cellular lines, with the capacity to modulate tumor cell migration and invasion [24]. In the present study, our researchers uncovered a remarkable finding: the combinatorial treatment of apigenin and cisplatin effectively diminished the expression levels of KRT23. By crafting an overexpression plasmid for KRT23 and introducing it into CRC cells, we observed a partial reversal of the inhibitory effects imparted by the apigenin-cisplatin combination. This suggests that the combined action of apigenin and cisplatin may impede tumor cell proliferation by regulating KRT23 expression, a phenomenon that has not been documented in prior research. In 2020, an investigation into gene expression profiles of CRC tissue versus normal colon tissue unveiled a spectrum of differentially expressed genes and associated signaling pathways, including KRT23 and the Wnt/β-catenin signaling pathway, albeit without further exploration [25]. Our current study extends these findings by demonstrating that the combination of apigenin and cisplatin not only decreases KRT23 expression but also alters the expression of pivotal proteins within the Wnt/β-catenin signaling pathway, notably a reduction in β-catenin protein levels. This discovery hints at a potential interplay between KRT23 and the Wnt/β-catenin signaling pathway. Furthermore, our examination of the impact of KRT23 overexpression on CRC cells revealed a corresponding alteration in β-catenin protein expression. This underscores the notion that variations in KRT23 expression levels can elicit changes in β-catenin protein expression, thereby suggesting a functional link between these two entities.

Currently, multiple studies have indicated that enhanced glycolysis significantly promotes tumor progression. Targeting glycolysis is very attractive for tumor therapy [26]. Currently, a variety of drugs targeting glucose transporters, hexokinases, and lactate dehydrogenase in the process of glycolysis have entered clinical trials [27]. In the current

study, it was found that the combination of apigenin and cisplatin can inhibit two key proteins (HK-2 and Glut1) in the glycolysis process, indicating that apigenin combined with cisplatin may participate in regulating the occurrence and development of colorectal cancer by regulating the glycolysis process.

It is well-established that inflammation arises as the body's response to pathogens and physical or chemical insults, aimed at eliminating damage and restoring physiological homeostasis. In the context of chronic inflammation, intricate interactions among cytokines, chemokines, and oxidative stress, induced by inflammatory cells and damaged parenchymal cells, may ultimately culminate in oncogenesis [28]. For colorectal cancer (CRC), the incidence of malignancy is intimately tied to the severity of colitis. Inflammatory cytokines, a class of small-molecule proteins with extensive biological activities, participate in inflammatory responses by being synthesized and secreted by certain immune cells in response to diverse stimuli. Among the myriad of inflammatory cytokines, TNF-α, IL-6, IL-8, and IL-10 occupy pivotal roles [29]. In this study, following an investigation into the effects of apigenin combined with cisplatin on the proliferation, apoptosis, and metastasis of CRC cells, we further examined alterations in the levels of TNF-α, IL-6, and IL-8. Our findings revealed that, in comparison to the control group, apigenin was capable of reducing the levels of these inflammatory cytokines, whereas cisplatin had the opposite effect, elevating their concentrations. These results indicate that both apigenin and cisplatin exert influences on the levels of inflammatory cytokines, hinting that the incorporation of a specific dose of apigenin during cisplatin-based chemotherapy for CRC may mitigate the patient's inflammatory response and potentially reduce chemotherapy resistance.

In contrast to other flavonoids, apigenin not only exhibits anticancer properties but has also been confirmed in prior research to possess the distinctive attribute of low toxicity. Studies conducted on normal cellular populations have failed to uncover any disruptive effects of apigenin on cellular growth [30]. Rather, it has been shown to bolster cellular protection and quell inflammatory responses. In investigations exploring the combination of apigenin with chemotherapy agents for the treatment of tumor cells, numerous studies have highlighted that apigenin augments the anticancer efficacy of these drugs, diminishes their adverse effects, inhibited drug resistance, and preserves the integrity of healthy cells [31]. Furthermore, these combinatorial therapies exhibit better therapeutic effects against diverse cancer cell types, wherein apigenin sensitizes chemotherapy drugs through distinct pathways compared to monotherapy, notably by markedly reducing the overexpression of genes, AKT phosphorylation, and the NF-κB signaling pathway [32]. Meanwhile, compared to individual chemotherapy drugs, combination therapy significantly induced apoptosis of treated cells [33, 34]. This was similar to the research in this article. In summary, the above studies could indicate that the combination therapy of apigenin could inhibit or reduce the resistance and toxicity of certain chemotherapy drugs.

A small amount of research has also explored the relationship between apigenin and cisplatin. For example, in 2017, Liu et al.'s study showed that as a tumor suppressor, p53 has an important role in tumour progression and functions as a critical tumor suppressor, and when apigenin and cisplatin are used together, they can inhibit tumor cell proliferation and are closely related to the expression level of p53 protein [35]. However, current research has not considered the effect of p53 protein, which will be the direction

we need to study in the future. In addition, in the study of cisplatin induced kidney injury in mice, it was found that apigenin has a protective effect on the kidneys and is related to oxidative stress [36]. However, current research has not detected changes in the content of oxidative stress factors (glutathione peroxidase and superoxide dismutase) in cell supernatant, and it is unclear whether apigenin and cisplatin will affect the content of oxidative stress factors in the treatment of colorectal cancer. In addition to these, there are also some limitations that deserve attention. For example, current research lacks knockdown/silencing experiments on the KRT23 gene to fully confirm its regulatory mechanism in the treatment of CRC with apigenin and cisplatin. At the same time, this study can also increase the sample size and conduct some clinical validations. In future research, these need to be further elaborated to fully elucidate the viewpoints of this study. This will provide a scientific theoretical basis for the drugs used to treat colorectal cancer.

In conclusion, our results suggested that API combined with DDP suppressed the progression of colorectal cancer by targeting the Glycolysis/KRT23/Wnt/β-catenin signaling pathway. Furthermore, more clinical studies were needed to verify whether API could be used as an adjunct agent for CRC chemotherapy.

Supplementary Information

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Supplementary Material 1. Fig S1 API combined with DDP to inhibit CRC cell migration and invasion. A Transwell migration assays were used to determine the migration ability of CRC cells. B wound healing assays were performed to estimate the migration ability of CRC cells. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups.

Supplementary Material 2. Fig S2 Overexpression of KRT23 gene could restore the migration and invasion abilities inhibition of API combined with DDP on colorectal cancer cells. A The migration abilities of CRC cells was assessed by transwell assay. B The invasion abilities of CRC cells was detected by transwell assay. Each experiment was repeated at least 3 times. Statistical analysis was executed through the t-test or ANOVA. * indicate statistically significant differences between groups.

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Author contributions
Yuesheng Gong, Lei Dong and Feng Yang conceived and designed the experiments. Feng Yang, Xue Wang, Panpan Jiao, Mengjie Kang, Wei Zhang, Jucai Song and Qianqian Guo performed the experiments. Liangzhu Si, Shuhan Zhang, linshan Luo and Mei Huang performed the data analyses. Feng Yang, Xue Wang, Wei Zhang and Yongwei Li drafted and revised the manuscript. Lei Dong finalized the manuscript. Yuesheng Gong, Yongwei Li and Lei Dong confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

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Availability of data and materials
The data generated in the present study may be requested from the corresponding author.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou Anorectal Hospital, and were performed in accordance with the ARIVE guidelines and National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Once the tumor volume reaches or exceeds 1000 cubic millimeters (mm^3), the mice were immediately euthanized to prevent unnecessary suffering.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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