

# World Journal of *Gastrointestinal Oncology*

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**ABOUT COVER**

Editorial board member of *World Journal of Gastrointestinal Oncology*, Dr. Cao is an Assistant Research Fellow at the Second Affiliated Hospital of Soochow University in Suzhou, China. Having received his Bachelor's degree from Soochow University in 2006, Dr. Cao undertook his postgraduate training at Soochow University, receiving his Master's degree in 2009 and his PhD in 2015. He works in the Department of Pathology, the Second Affiliated Hospital of Soochow University, Soochow University, where his ongoing research interests involve the molecular pathological mechanisms of malignant tumors, particularly in relation to oncogenesis, radiation resistance and cellular signal transduction involving tumors of the digestive system. To date, he has published 16 SCI papers as corresponding author or first author. (L-Editor: Filipodia)

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## Basic Study

## Interleukin-1 receptor antagonist enhances chemosensitivity to fluorouracil in treatment of Kras mutant colon cancer

Yan Yan, Hong-Wei Lin, Zhuo-Nan Zhuang, Ming Li, Sen Guo

**ORCID number:** Yan Yan 0000-0002-2511-0965; Hong-Wei Lin 0000-0003-3648-6949; Zhuo-Nan Zhuang 0000-0002-0881-2631; Ming Li 0000-0003-0970-1156; Sen Guo 0000-0003-0175-3963.

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**Yan Yan,** Department of Operating Room, Qilu Hospital, Shandong University, Jinan 250012, Shandong Province, China

**Hong-Wei Lin, Zhuo-Nan Zhuang,** Department of Gastrointestinal Surgery, Beijing Tsinghua Changgung Hospital School of Clinical Medicine, Tsinghua University, Beijing 102200, China

**Ming Li,** Department of General Surgery, Zouping Traditional Chinese Medicine Hospital, Zouping 256200, Shandong Province, China

**Sen Guo,** Department of General Surgery, Qilu Hospital, Shandong University, Jinan 250012, Shandong Province, China

**Corresponding author:** Sen Guo, PhD, Adjunct Professor, Department of General Surgery, Qilu Hospital of Shandong University, No. 107 Wenhuxi Street, Jinan 250012, Shandong Province, China. [guosen4321@163.com](mailto:guosen4321@163.com)

## Abstract

### BACKGROUND

Kras mutant colon cancer shows abnormal activation of the nuclear factor kappa-B (NF- $\kappa$ B) pathway, resulting in the proliferation of tumor cells. Treatment with fluorouracil (5-FU) might not achieve the expected inhibition of proliferation of malignant cells based on the fluorouracil-induced activation of the NF- $\kappa$ B pathway.

### AIM

To detect whether interleukin (IL)-1 receptor antagonist (IL-1RA) could increase the chemosensitivity to 5-FU by decreasing the activation of the NF- $\kappa$ B pathway and reducing the proliferation of colon cancer cells.

### METHODS

Western blot analysis was performed to detect the persistent activation of the NF- $\kappa$ B pathway in colon cancer cell lines. Reverse transcription-polymerase chain reaction was used to detect the IL-1RA-reduced expression levels of IL-6, IL-8, IL-17, IL-21 and TLR4 in colon cancer cell lines. We used a xenograft nude mouse model to demonstrate the downregulation of the NF- $\kappa$ B pathway by blocking the NF- $\kappa$ B-regulated IL-1 $\alpha$  feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting tumor cell growth.

### RESULTS

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IL-1 receptor antagonist could decrease the expression of IL-1 $\alpha$  and IL-1 $\beta$  and downregulate the activity of the NF- $\kappa$ B pathway in Kras mutant colon cancer cells. Treatment with 5-FU combined with IL-1RA could increase the chemosensitivity of the SW620 cell line, and decreased expression of the TAK1/NF- $\kappa$ B and MEK pathways resulted in limited proliferation in the SW620 cell line.

### CONCLUSION

Adjuvant chemotherapy with IL-1RA and 5-FU has a stronger effect than single chemotherapeutic drugs. IL-1RA combined with fluorouracil could be a potential neoadjuvant chemotherapy in the clinic.

**Key words:** Colon carcinoma; Chemotherapy; Nuclear factor kappa-B; Interleukin-1; Proliferation; Fluorouracil

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**Core tip:** A feedback loop between the upregulated nuclear factor kappa-B (NF- $\kappa$ B) pathway and interleukin (IL)-1 leads to the proliferation of cancer cells. Fluorouracil (5-FU), a chemotherapy drug used to treat colon carcinoma cells, can activate the NF- $\kappa$ B pathway and lead to chemotherapy resistance. IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than single 5-FU treatment due to the blockade of IL-1. This report could provide an adjuvant chemotherapy strategy for the clinic and provide a theoretical basis for neoadjuvant chemotherapy.

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## INTRODUCTION

Colorectal cancer is the primary cause of death among gastrointestinal cancers and the third most common cancer worldwide<sup>[1,2]</sup>. Nearly 50% of patients with recurrent colon cancer undergo colon cancer procedures and chemotherapy<sup>[3]</sup>, and it has been indicated that the current chemotherapy regimen may not be effective in controlling the recurrence and metastasis of the tumor<sup>[4]</sup>. Systemic toxicity and drug resistance of tumor cells are two major problems in cancer chemotherapy<sup>[5]</sup>. Therefore, various studies have explored how to reduce the toxicity of conventional chemotherapeutic drugs and increase the chemical sensitivity to achieve better curative effects of chemotherapy and gain more benefits for patients with colon cancer<sup>[6,7]</sup>.

Fluorouracil (5-fluorouracil, 5-FU) is the first-choice drug for various chemotherapy regimens of colon cancer in recent decades<sup>[8]</sup>. Even the current classic chemotherapy plans, including the FOLFOX regimen and FOLFIRI regimen, contain 5-FU as a component for colon cancer treatment. This drug can inhibit the synthesis of adenylate synthetase and interfere with the synthesis of DNA in tumor cells. The growth of cells remained at a low level, and cell apoptosis was increased<sup>[9]</sup>. However, the effect of 5-FU is not ideal due to the chemoresistance in colon carcinoma patients treated with 5-FU<sup>[10]</sup>. The clinical benefit of colon cancer patients is considered to be limited, especially for those with advanced tumors<sup>[11]</sup>. Adjuvant chemotherapy has been studied to overcome the chemoresistance to 5-FU in colon cancer.

Kras mutant colon carcinoma shows persistent activation of the nuclear factor kappa-B (NF- $\kappa$ B) pathway, which promotes the proliferation and metastasis of tumor cells<sup>[12]</sup>. The persistently activated NF- $\kappa$ B pathway could promote chemoresistance to 5-FU in colon cancer treatment<sup>[13]</sup>. NF- $\kappa$ B is a transcription factor protein that includes five subunits: Rel (cRel), p65 (RelA, NF- $\kappa$ B3), RelB, and p50 (NF- $\kappa$ B1)<sup>[14]</sup>. The high expression of NF- $\kappa$ B is related to inflammatory factors and is closely related to cell

growth and proliferation<sup>[15-17]</sup>. In tumor biology, the NF- $\kappa$ B pathway is highly active with high expression in various tumor cells<sup>[18]</sup>. It was found that 5-FU could increase the phosphorylation of P65 in colon cancer cells, which increased the chemotherapy resistance to 5-FU in clinical treatment<sup>[19,20]</sup>. However, downregulating the NF- $\kappa$ B pathway increased the chemosensitivity to 5-FU in colon cancer chemotherapy<sup>[21]</sup>.

Our previous studies have shown that NF- $\kappa$ B remains persistently activated in *Kras* mutant pancreatic cancer<sup>[22,23]</sup>, which is closely related to the high expression of interleukin (IL)-1 $\alpha$ <sup>[24]</sup>. IL-1 $\alpha$  can increase the activity of the NF- $\kappa$ B pathway by upregulating AP-1 in pancreatic cancer cells<sup>[25]</sup>. Similarly, the inhibition of NF- $\kappa$ B activity also decreased the expression of IL-1 in pancreatic cancer cells. IL-1 and NF- $\kappa$ B show a cyclic relationship, which leads to persistent activation of NF- $\kappa$ B in tumor cells<sup>[26]</sup>. In *Kras* and *p53* mutant mice, we found that the NF- $\kappa$ B activity was downregulated by inhibiting the IL-1 receptor, which could effectively slow tumor growth. Other studies have shown that an NF- $\kappa$ B inhibitor had proapoptotic effects on colon cancer cells following IL-6 stimulation<sup>[27]</sup>. The aim of this study was to assess whether treatment with 5-FU combined with IL-1 receptor antagonist can increase the chemosensitivity to 5-FU by decreasing the activation of the NF- $\kappa$ B pathway and reducing the proliferation of colon cancer cells. The results obtained will provide a theoretical basis for clinical adjuvant chemotherapy.

## MATERIALS AND METHODS

### Cell lines, reagents, and animals

The normal epithelial cell line (NCM460 cell line) and the human colon carcinoma cell lines (including COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116) were purchased from Nanjing Purisi Biotechnology Company (Jiangsu, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM Caisson Laboratories, Inc.).

TRIzol (American Invitrogen 15596-026); ethanol, chloroform, isopropanol (National Drug Group); cDNA first chain synthesis kit (United States Thermo Fisher K1622); and SYBR Premix Ex Taq II (Japanese TaKaRa RR820A) were used in this study. Primer design was performed by Nanjing Golden Srey Technology Co., Ltd. Substance synthesis and PAGE primer purification were also performed. The drug 5-FU was purchased from Thermo Biocompany. IL-1RA was purchased from Nanjing Purisi Biotechnology Company.

Thirty male athymic nude mice (NCI-nu), which were 4-6 weeks old and weighed approximately 24.9-33.0 g, were purchased from Nanjing Purisi Biological Company. All mice were housed and treated at Shandong University in accordance with the guidelines of The Animal Care and Use Committee, which provided the license number SYNK (Lu) 2019-0005, and the scope of application: Barrier environment and SPF level (dogs, rabbits, rats, and mice). SW620 colon cancer cells were harvested in PBS with 20% Matrigel (Fisher Scientific). Then, all nude mice were subcapsularly injected with SW620 colon cancer cells ( $1.0 \times 10^6$  cells in 50  $\mu$ L of PBS) in the subcutaneous tissue of the back. The effect of chemotherapy was observed in 15 nude mice with tumor loads that were euthanized by carbon dioxide inhalation (the flow rate of CO<sub>2</sub> used for euthanasia increased from 0% to 20% of the chamber volume per minute). Lack of breath and discoloration of the eyes were observed in all nude mice. The flow of carbon dioxide was maintained for a minimum of 1 min after respiratory arrest, and the tumor tissues were dissected (cervical dislocation was used for the approved secondary physical method of euthanasia). All mice were evaluated every 3 d to observe tumor growth during the 3-wk treatment. Tumor volume was determined as follows:  $V = (\text{length} \times \text{width}^2)/2$ . If multiple tumors were present, the final result was the sum of the measured results of each single tumor. The limited diameter of the tumor was 3 cm, which measured a single tumor or the sum of multiple tumors.

The survival time was observed in the other 15 nude mice, which died due to cachexia or overloaded tumors more than 3 cm in diameter. The groups were as follows: Control group (5 nude mice with PBS treatment), 5-FU group (5 nude mice with 5-FU treatment), and 5-FU and IL-1RA group (5 nude mice with 5-FU and IL-1RA treatment). For *in vivo* studies, 1.5 mg of intraperitoneal rhIL-1RA diluted with PBS was used to treat the nude mice (daily, 3 wk), and 20 mg/kg of intraperitoneal 5-FU diluted by PBS was used to treat the nude mice (twice a week, 3 wk).

### Western blot assay

Cell lysates were extracted from cells with radioimmunoprecipitation assay protein

lysate buffer. The cellular extracts were boiled for 5 min to denature the protein. A total of 30 µg of protein was loaded into each well and separated on a gel. Then, the protein samples were transferred to a polyvinylidene fluoride (PVDF) membrane for 1 h at 300 mA. The PVDF membrane was blocked with 5% skim milk powder in 0.1% TBST for 1 h and incubated overnight with primary antibodies at 4°C. The primary antibodies against phosphorylated p65, p65, phosphorylated TAK1, TAK1, phosphorylated MEK, and MEK were purchased from Nanjing Puruisi Biotechnology Company and diluted 1:500. The primary antibody against IL-1α was purchased from ABcam Biotechnology Company and diluted 1:200. The secondary antibodies and β-actin antibody were purchased from ABcam Biotechnology Company.

### **Reverse transcription-polymerase chain reaction**

The optical density (OD) values of RNA samples extracted from cells were measured at 260 nm and 280 nm. RNA concentration was calculated as  $OD_{260} \times \text{dilution factor} \times 0.04 \mu\text{g}/\mu\text{L}$ . The range of  $OD_{260/280}$  was 1.8 to 2.1, indicating a high purity of the extracted RNA. Then, the samples were mixed with nuclease-free enzyme, oligo dT (18), and nuclease-free double-distilled water to the total volume. Mixed RNase inhibitor, reaction buffer, dNTPs, DTT (1 M), reverse transcriptase (AMV), and nuclease-free double-distilled water were added in reverse transcription-polymerase chain reaction (RT-PCR) tubes. After the cDNA reaction, the samples were subjected to amplification. The cycle conditions were as follows: Denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 35 s at 72 °C. A total of 32 cycles were performed. The sequence information is as follows: hIL-1α-F, 3'-TCCCCAGGGACCTCTCTCTA-5' and hIL-1α-R, 3'-GAGGGTTTGCTACAACATGGG-5'; hIL-1β-F, 3'-TCGCCAGTGAAATGATGGCT-5' and hIL-1β-R, 3'-TGGAAGGAGCACTTCATCTG; hIL-6-F, 3'-TCAATATTAGAGTCTCAACCCCA-5 and hIL-6-R 3'-GAAGGCGCTTGTGGAGAAGG-5; hIL-8-F, 3'-GCTCTGTGTAAGGTG CAGTT-5' and hIL-8-R, 3'-ACCCAGTTTCCTTGGGGTC-5'; hIL-17-F, 3'-TGGAATCTCCACCGCAATGA-5' and hIL-17-R, 3'-GCTGGATGGGGACAGAGTTC-5'; hIL-21-F, 3'-ACACAGACTAACATGCCCTTCA-5' and hIL-21-R, 3'-ACCGTGAGTAACTAAGAAGCAA-5'; TLR4-F, 3'-GGTCAGACGGTGATAGCGAG-5' and hTLR4-R, 3'-TTTAGGGCCAAGTCTCCACG-5'; hP65-F, 3'-ACAACAACCCCTTCCAAGAAGA-5' and hP65-R, 3'-TCACTCGGCAGATCTTGTG-5'.

### **Gene silencing assay**

After being treated with P65-siRNA for 48 h for interference, the SW4690 cell line was assessed for the RNA and protein levels of the target gene by RT-PCR and/or Western blot assays. The P65-siRNA oligo package was purchased from Suzhou Gemma Gene Biotechnology Company. The information of two basic P65-siRNAs is as follows: (1) siRNA1: Sense, 5'-GGCGAGAGGAGCACAGAUACC-3' and antisense, 5'-UAUCUGUGCUCUCCUCGCCUG-3'; and (2) siRNA2: Sense, 5'-CCCACGAGCUU GUAGGAAAGG-3' and antisense, 5'-UUUCCUACAAGCUCGUGGGGG-3'. The sequence of siRNA scramble (GenePharma Company, Shanghai) is: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACG UUCGGAATT-3'. The information of two basic P65-siRNAs is as follows: Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCG GAGAATT-3'.

### **Cell proliferation assay**

For the MTT assay, the cell suspension was seeded in each well of 96-well plates. After 12 h, attached cells were treated with various doses of 5-FU or/and IL-1RA. The cells were incubated in 4.5% CO<sub>2</sub> at 37 °C for 1, 2, 3, 4, and 5 d, 10 µL of MTT solution (5 mg/mL) was added to crystallize the cells for 4 h, and 150 µL of DMSO was added for 10 min to oscillate the cells. The absorbance value was measured at 490 nm. In the colony formation assay, DMEM-diluted cell suspension was inoculated in a 6-well culture dish containing 10 mL of 37 °C incubation medium at a density of 300 cells per well. After 12 h, attached cells were treated with various doses of 5-FU or/and IL-1RA. The cells were incubated in a cell incubator for 2 wk, and then, the colonies were immobilized with formalin (Sigma-Aldrich) within 30 min and stained with crystal violet (Sigma-Aldrich) within 1 h.

### **Statistical analysis**

Commercially available SPSS version 19.0 software (Chicago, IL, United States) and GraphPad Prism software (La Jolla, CA, United States) were used for statistical

analyses. An unpaired *t*-test (one-tailed) was used to analyze the differences between groups. One-way ANOVA was used to analyze the differences among multiple groups. The log-rank test was used to analyze the differences in survival time between groups. The Bonferroni test was used following ANOVA for multiple comparisons. *P* < 0.05 was considered statistically significant.

## RESULTS

### ***The Kras gene persistently activates the NF-κB pathway in colon carcinoma cells***

The abnormal activity of cancer cells was determined by the NF-κB pathway, which led to the proliferation of malignant cells, and this pathway could be persistently activated by the *Kras* mutant gene targeting TAK1 and AP1<sup>[26]</sup>. In colon carcinoma cell lines, including COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116, the activity of the *Kras* gene remained high compared with that in NCM460, a normal epithelial cell line (Figure 1A). *Kras* gene mutation in colon cancer also resulted in excessive proliferation of malignant cells through persistent activation of the NF-κB pathway<sup>[28]</sup>. The expression of phosphorylated P65 in the COLO205, SW620, and HCT116 cell lines was significantly higher than that in the NCM460 cell line, as shown by Western blot assays (Figure 1B and C). The expression of IL-1α in the COLO205, SW620, and HCT116 cell lines remained at a high level compared with that in the NCM460 cell line (Figure 1D and E).

### ***Interleukins are expressed at high levels in NF-κB-activated colon carcinoma cells***

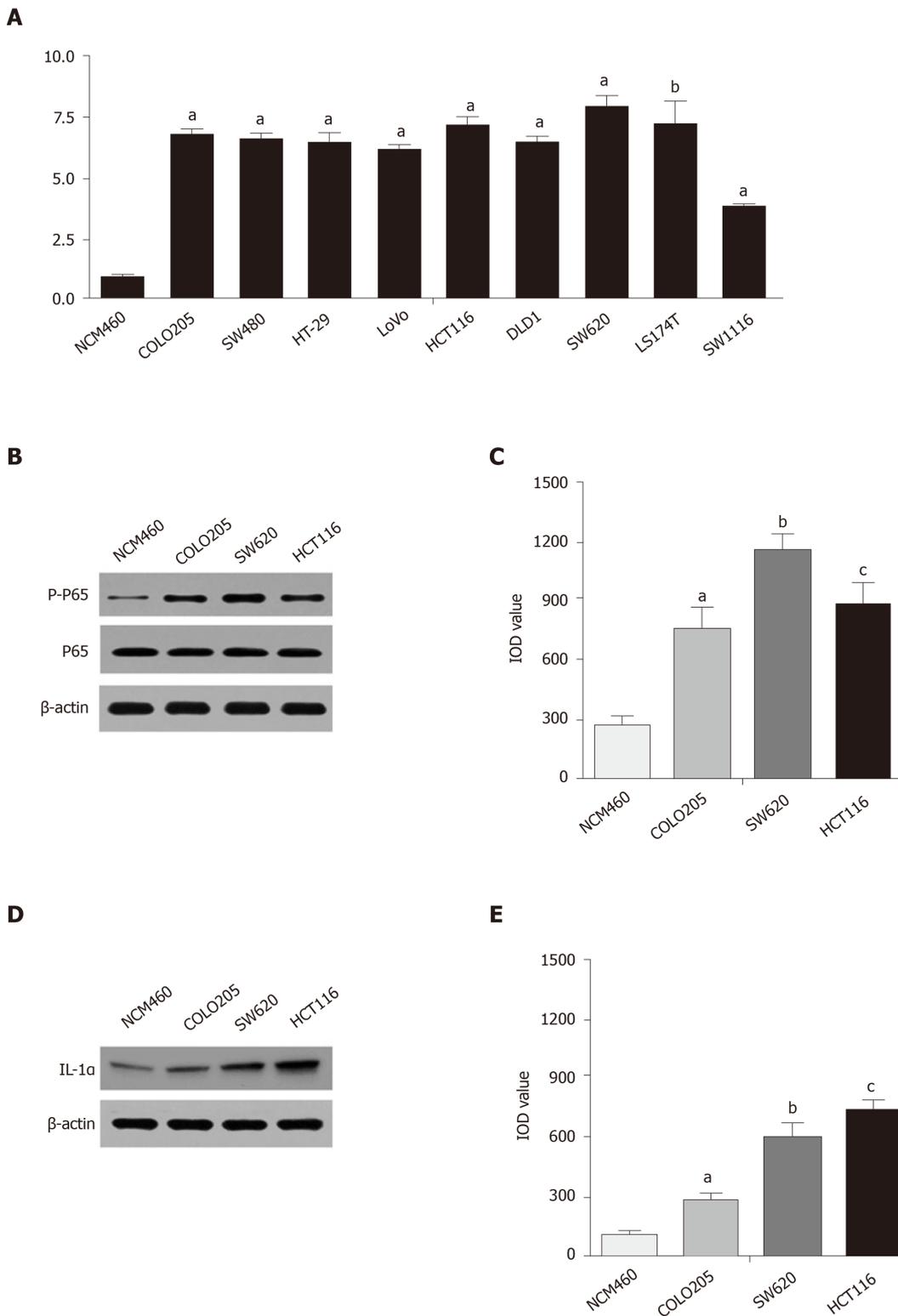
Previous studies focused on IL-6 for growth inhibition of colon cancer cells<sup>[29]</sup>. In this study, IL-1α and IL-1β were targeted to detect their expression, which remained at a high level in colon carcinoma cells with persistent activation of the NF-κB pathway (Figure 2A and B). The continuous activation of the NF-κB pathway was confirmed to increase the expressions of IL-6, IL-8, IL-17, IL-21, and TLR4 in colon carcinoma cell lines (Figure 2C-G). The activity of the NF-κB pathway was inhibited to observe whether it could decrease IL-1α and IL-1β in the colon carcinoma cell line. The SW620 cells were treated with siRNA to downregulate the activity of the NF-κB pathway. The mRNA level of P65 was decreased by interference with siRNA1 and siRNA2 in the SW620 cell line, as shown by RT-PCR assays, compared with that of the untransfected SW620 cells (Figure 3A and B). The mRNA levels of IL-1α and IL-1β were also significantly decreased with siNF-κB interference in the SW620 cell line (Figure 3C and D). The expression levels of IL-6, IL-8, IL-17, IL-21, and TLR4 were reduced in the colon cancer cell lines after siRNA interference in the NF-κB pathway (Figure 3E-I). The results suggested that IL-1 is closely related to the NF-kappa B pathway in the SW620 cell line of *Kras* mutant colon carcinoma.

### ***Inhibition of IL-1 reduces the abnormal activation of the NF-κB pathway induced by 5-FU and decreases the high expression of TAK1 and MEK***

Abnormal activity of the NF-κB pathway was found in SW620 cells when they were treated with 5-FU chemotherapy. After 5-FU treatment, SW620 cells expressed high levels of phosphorylated TAK1 and phosphorylated MEK, which could explain the unexpected proliferation of drug-resistant malignant cells after chemotherapy (Figure 4A). Western blot assays showed that the expression of phosphorylated TAK1 and phosphorylated MEK was significantly higher in the SW620 cell line treated with 5-FU than in the untreated SW620 cell line (Figure 4B and D). This phenomenon in the SW620 cell line could be inhibited by 5-FU combined with IL-1 receptor antagonist, which decreased the phosphorylation of P65 and had an inhibitory effect on the phosphorylation of TAK1 and MEK. Western blot assays showed that the expression levels of phosphorylated TAK1 and phosphorylated MEK were significantly lower in the 5-FU and IL-1 receptor antagonist treatment groups than in the 5-FU treatment group (Figure 4B-D).

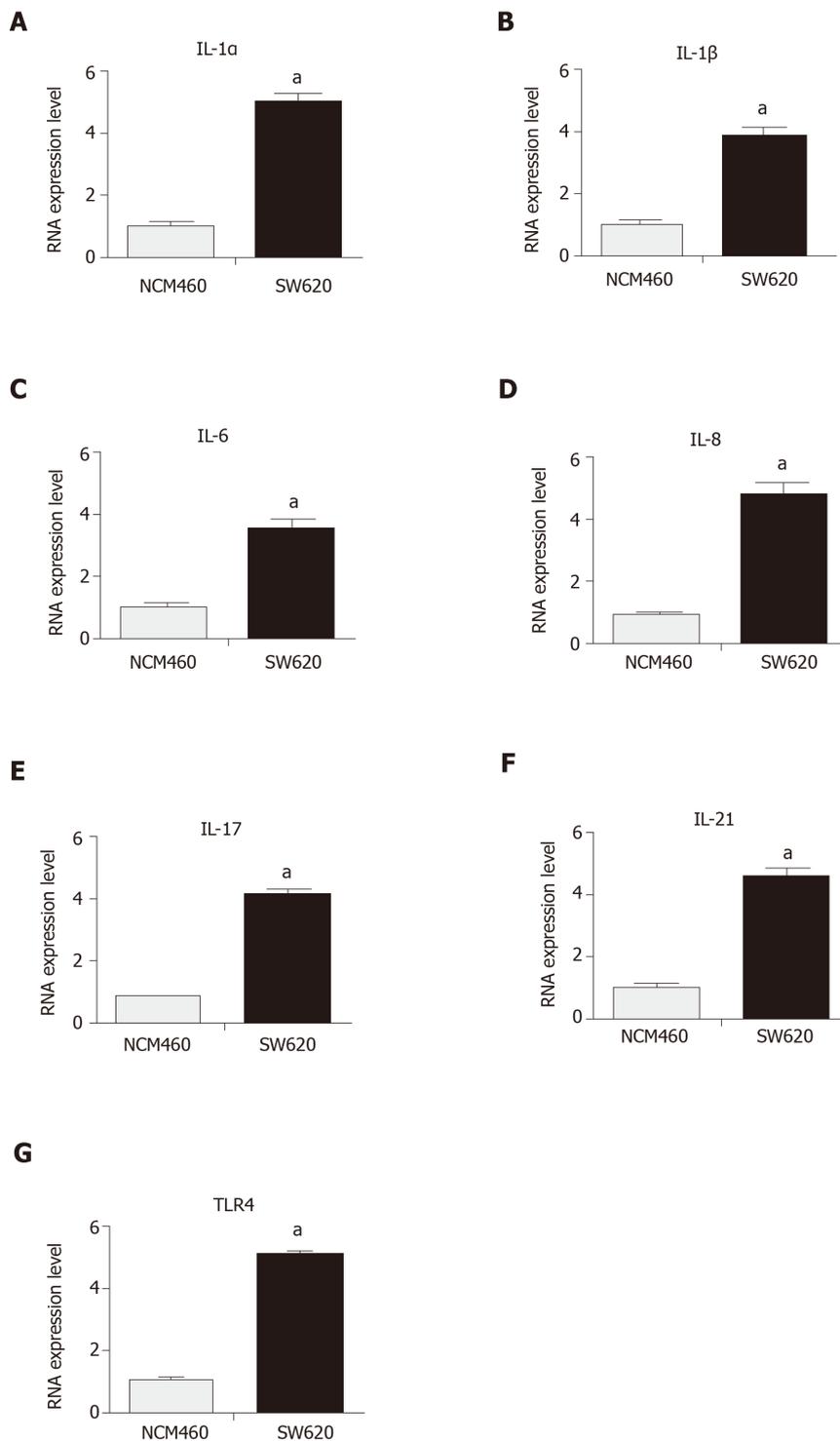
### ***IL-1RA combined with 5-FU inhibits the NF-κB pathway to decrease the proliferation of colon carcinoma cells***

As a chemotherapeutic agent for colon cancer, 5-FU is widely used in clinical treatment, but side effects and drug resistance can occur. First, 6.25, 12.5, and 25 mg/mL of 5-FU was used to treat the SW620 and HCT116 cell lines, and the trend of the cell growth curve was observed at 96 h by MTT assays. IL-1RA combined with 5-FU was more effective in inhibiting the proliferation of the SW620 and HCT116 cell



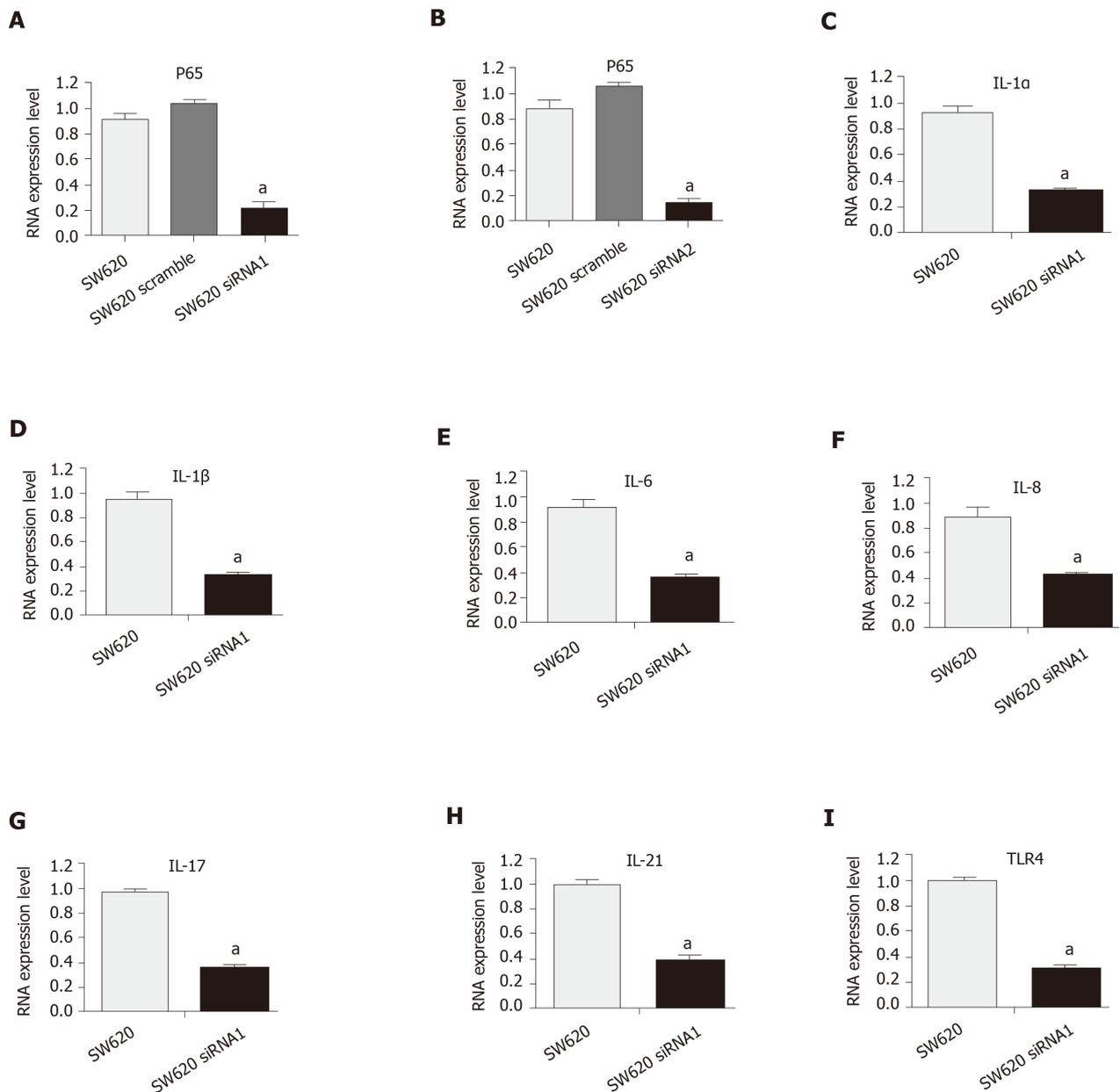
**Figure 1** Expression of phosphorylated nuclear factor kappa-B in Kras mutant colon carcinoma cell lines. A: Reverse transcription-polymerase chain reaction assays showed that the expression of the Kras gene in the COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116 cell lines was high. The expression level of Kras in the COLO205 cell line was significantly higher than that in NCM460 (<sup>a</sup> $P < 0.0001$ , <sup>b</sup> $P = 0.0001$  vs NCM460; comparison of multiple groups:  $P < 0.0001$ ); B and C: Western blot assays showed that the expression levels of phosphorylated nuclear factor kappa-B (NF-κB) in the COLO205, SW620, and HCT116 cell lines were increased compared with that of NCM460 (COLO205 vs NCM460, <sup>a</sup> $P = 0.0467$ ; SW620 vs NCM460, <sup>b</sup> $P = 0.0050$ ; HCT116 vs NCM460, <sup>c</sup> $P = 0.0177$ ; comparison of multiple groups:  $P = 0.0081$ ); D and E: Western blot assays showed that the expression of interleukin-1α was higher in the COLO205, SW620, and HCT116 cell lines than the NCM460 cell line (COLO205 vs NCM460, <sup>a</sup> $P = 0.0427$ ; SW620 vs NCM460, <sup>b</sup> $P = 0.0100$ ; HCT116 vs NCM460, <sup>c</sup> $P = 0.0024$ ; comparison of multiple groups:  $P = 0.0019$ ).

lines than 5-FU alone. The results showed that 200 mg/mL of IL-1RA combined with 12.5 mg/mL of 5-FU could significantly inhibit cell growth (Figure 5). IL-1RA



**Figure 2** Expression of interleukins in the activated nuclear factor kappa-B pathway in SW620 cells. A and B: Reverse transcription-polymerase chain reaction (RT-PCR) assay showed that the expression of interleukin (IL)-1 $\alpha$  and IL-1 $\beta$  was significantly higher in SW620 cells than in NCM460 cells (IL-1 $\alpha$ : SW620 vs NCM460,  $^aP = 0.0003$ ; IL-1 $\beta$ : SW620 vs NCM460,  $^aP = 0.0001$ ); C-G: RT-PCR assay showed that the expression of IL-6, IL-8, IL-17, IL-21, and TLR4 was significantly higher in SW620 cells than in NCM460 cells (IL-6: SW620 vs NCM460,  $^aP = 0.0005$ ; IL-8: SW620 vs NCM460,  $^aP = 0.0007$ ; IL-17: SW620 vs NCM460,  $^aP < 0.0001$ ; IL-21: SW620 vs NCM460,  $^aP < 0.0001$ ; TLR4-6 : SW620 vs NCM460,  $^aP < 0.0001$ ).

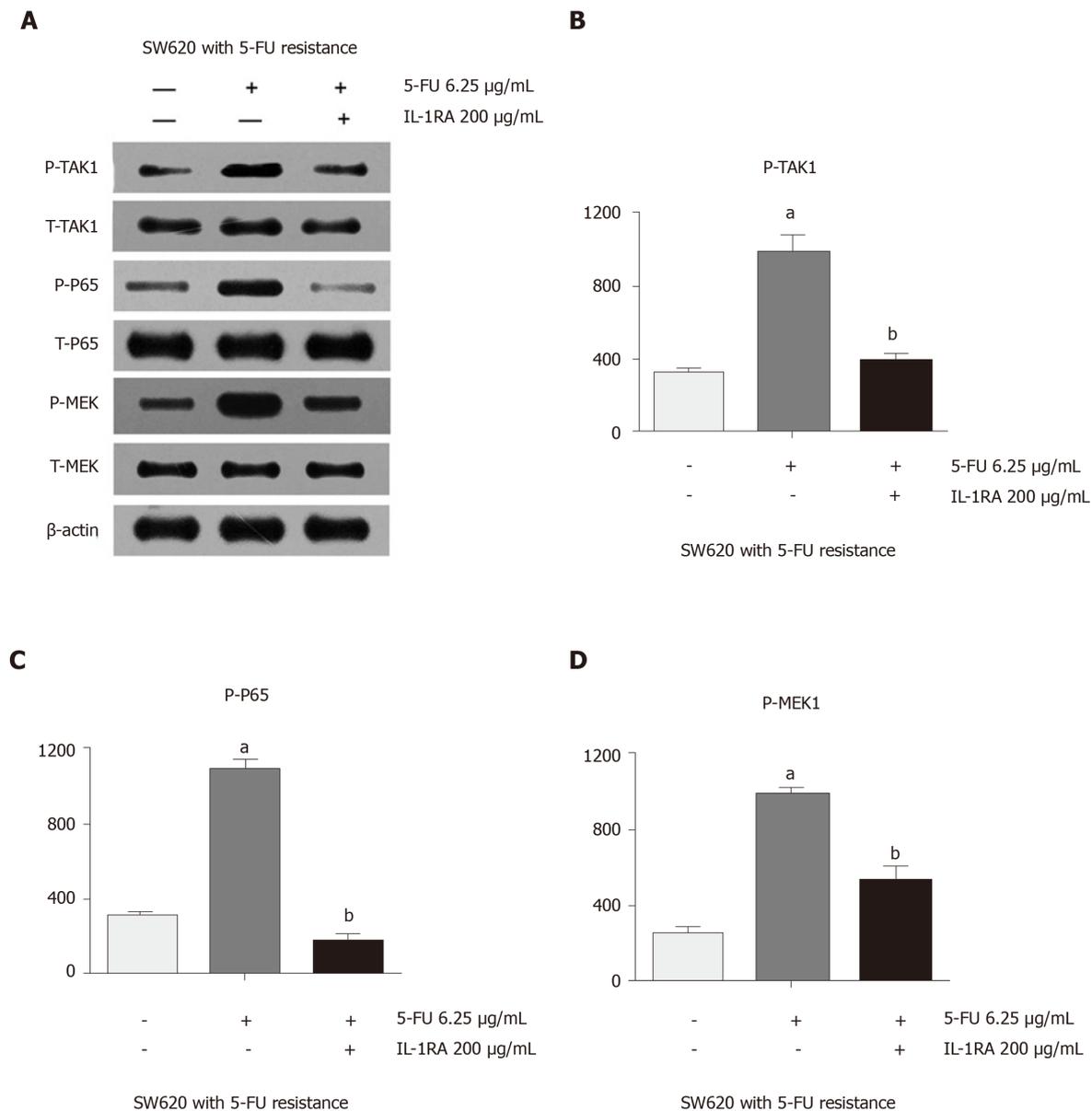
combined with 5-FU had a greater inhibitory effect on the monoclonal formation than single treatment. In clonogenicity assays, we used 200 mg/mL of IL-1RA and 6.25 mg/mL of 5-FU to treat the colon cancer cell line for 3 d. We found that the inhibitory effect of 200 mg/mL IL-1RA alone on the colony formation of tumor cells was weak, but IL-1RA combined with 5-FU had an obvious inhibitory effect on colony formation compared with 6.25 mg/mL 5-FU (Figure 6).



**Figure 3 Expression of interleukin-1α, interleukin-1β, and other interleukins in the siRNA-P65 SW620 cell line.** A: Reverse transcription-polymerase chain reaction (RT-PCR) assay showed the nuclear factor kappa-B (NF-κB) pathway was downregulated in the SW620 cell line after siRNA1 interference at 24 h (SW620 siRNA1 vs SW620, <sup>a</sup>*P* = 0.0003); B RT-PCR assay showed the NF-κB pathway was downregulated in the SW620 cell line after siRNA2 treatment at 24 h (SW620 siRNA2 vs SW620, <sup>a</sup>*P* = 0.0001); C: RT-PCR assay showed that the expression of IL-1α was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* = 0.0002); D: RT-PCR assay showed that the expression of IL-1β was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* = 0.0012); E: RT-PCR assay showed that the expression of IL-6 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* = 0.0012); F: RT-PCR assay showed that the expression of IL-8 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* = 0.0033); G: RT-PCR assay showed that the expression of IL-17 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* < 0.0001); H: RT-PCR assay showed that the expression of IL-21 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* = 0.0004); I: RT-PCR assay showed that the expression of TLR4 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 vs SW620, <sup>a</sup>*P* < 0.0001).

**Effectiveness of the NF-κB signaling blockade through IL1RA in enhancing the chemosensitivity to 5-FU in vivo**

We used a xenograft nude mouse model to demonstrate the downregulation of the NF-κB pathway by blocking the NF-κB-regulated IL-1α feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting tumor cell growth. The tumor size of the control group treated with PBS (100 μL/mouse) for 3 wk significantly increased compared to that of the experimental groups (Figure 7A and B).

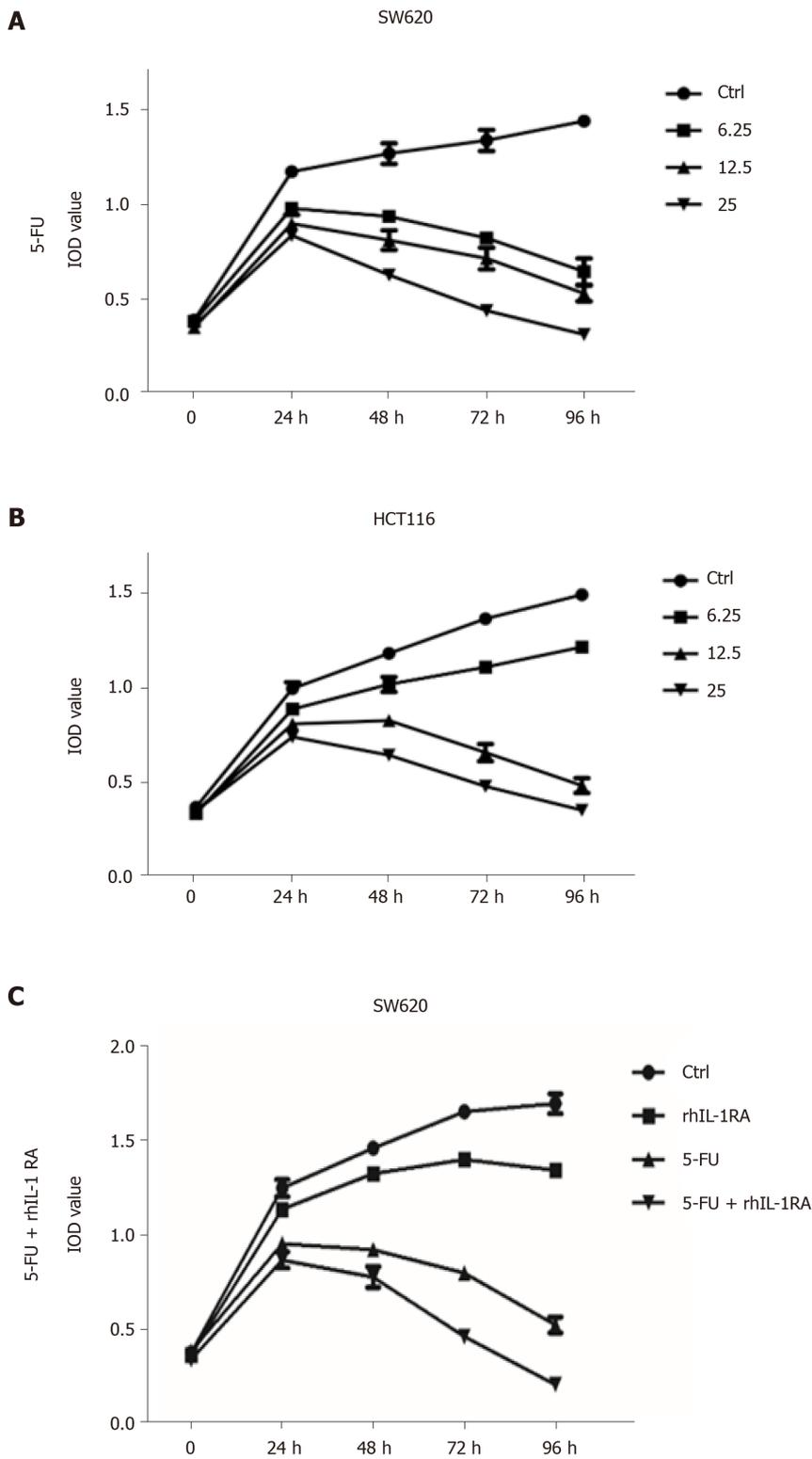


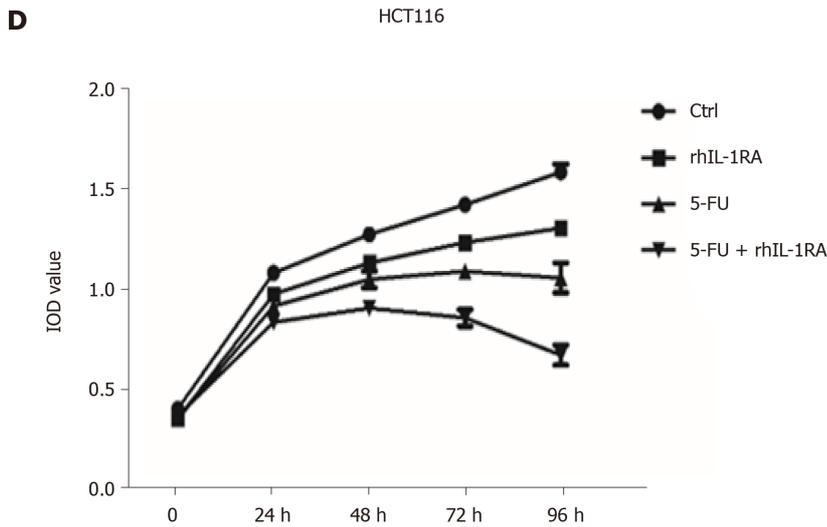
**Figure 4 Interleukin-1 RA could counteract the abnormally high expression of P-TAK1, P-P65, and P-MEK caused by fluorouracil in the SW620 cell line.** A: Western blot assay showed that the abnormally high expression of P-TAK1, P-P65 and P-MEK caused by fluorouracil (5-FU) was decreased by interleukin (IL)-1 RA treatment in the SW620 cell line; B: The abnormally high expression of P-TAK1 in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU vs the control, <sup>a</sup>*P* = 0.0199; 5-FU and IL-1RA vs 5-FU, <sup>b</sup>*P* = 0.0269); C: The abnormally high expression of P-P65 in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU vs the control, <sup>a</sup>*P* = 0.0048; IL-1RA vs 5-FU, <sup>b</sup>*P* = 0.0040); D: The abnormally high expression of P-MEK in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU vs the control, <sup>a</sup>*P* = 0.0019; IL-1RA vs 5-FU, <sup>b</sup>*P* = 0.0201).

After 3 wk of chemotherapy, the tumor weights of the nude mice treated with 5-FU and IL-1RA were significantly decreased compared with those in the single 5-FU treatment group (Figure 7C). In the 3-wk treatment, IL-1RA combined with 5-FU treatment limited the speed of tumor growth in the nude mice, according to the changes in tumor volume (Figure 7D) and diameter (Figure 7E). The survival time of the xenograft mouse model treated with chemotherapy was significantly longer than that of the control group treated with PBS (100 μL/mouse) (Figure 7F). IL-1RA combined with 5-FU treatment had a greater effect in extending the survival time of the xenograft tumor-bearing nude mice than 5-FU single therapy.

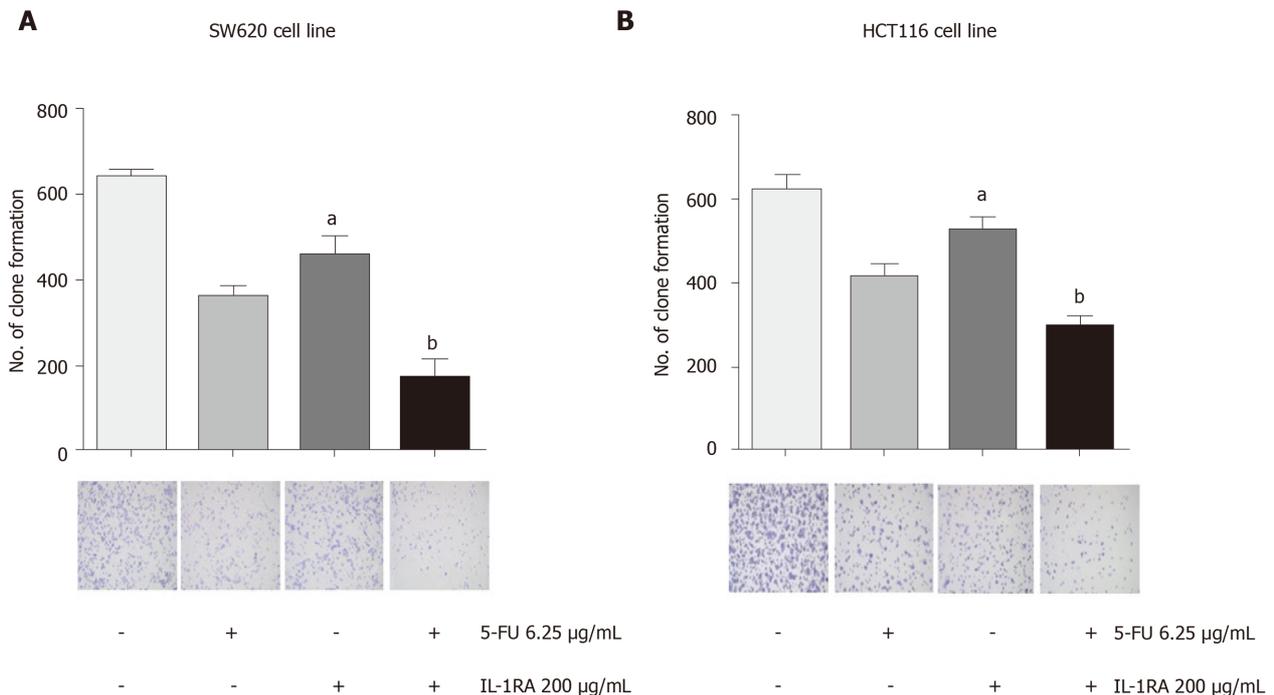
## DISCUSSION

Previous studies have found that 30%-40% of colon cancers have *Kras* gene mutations,



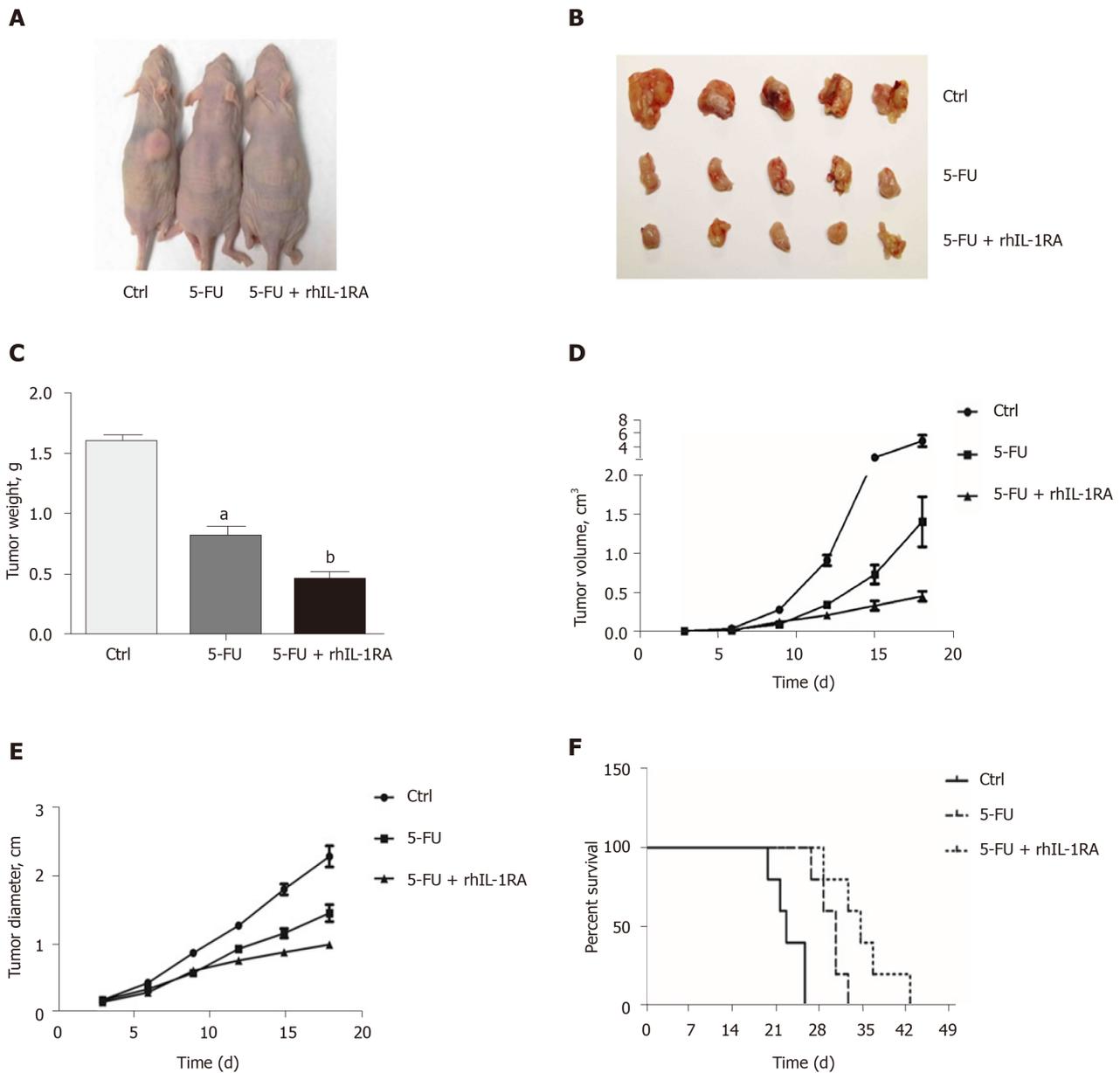


**Figure 5** Changes in the growth curves of the SW620 and HCT116 cell lines treated with interleukin-1RA and/or fluorouracil. A and B: MTT assays showed that the cell growth curves of the SW620 and HCT116 colon cancer cell lines treated with fluorouracil (5-FU) at concentrations of 6.25, 12.5, and 25 mg/mL exhibited a downward trend (SW620: 5-FU at 6.25 µg/mL for 96 h vs Ctrl,  $P < 0.0001$ ; HCT116: 5-FU at 6.25 µg/mL for 96 h vs Ctrl,  $P < 0.0001$ ); C: MTT assays showed that the cell growth curve of the SW620 colon cancer cell lines treated with interleukin (IL)-1RA was significantly different compared with the Ctrl (SW620: IL-1RA at 200 µg/mL for 96 h vs Ctrl,  $P = 0.0005$ ). The cell growth curve of the SW620 colon cancer cell lines treated with IL-1RA combined with 5-FU significantly decreased compared to that in the 5-FU group (SW620: 5-FU at 12.5 µg/mL and IL-1RA at 200 µg/mL for 96 h vs 5-FU at 12.5 µg/mL,  $P = 0.0003$ ); D: MTT assays showed that the cell growth curve of the HCT116 colon cancer cell lines treated with IL-1RA was significantly different compared with that of the Ctrl (HCT116: IL-1RA at 200 µg/mL for 96 h vs Ctrl,  $P = 0.0004$ ). The cell growth curve of the SW620 colon cancer cell lines treated with IL-1RA combined with 5-FU significantly decreased compared to that in the 5-FU group (HCT116: 5-FU at 12.5 µg/mL and IL-1RA at 200 µg/mL for 96 h vs 5-FU at 12.5 µg/mL,  $P = 0.0027$ ).



**Figure 6** Changes in colony formation in the SW620 and HCT116 cell lines treated with interleukin-1RA and/or fluorouracil. A: Colony formation assay showed that interleukin (IL)-1RA single drug treatment decreased the colony formation of SW620 cells, while this parameter significantly decreased in the SW620 cells treated with 6.25 mg/mL fluorouracil (5-FU) combined with 200 mg/mL IL-1RA compared with the untreated cells (5-FU vs Ctrl,  $P = 0.0027$ ; IL-1RA vs Ctrl,  $^aP = 0.0226$ ; 5-FU and IL-1RA vs IL-1RA,  $^bP = 0.0178$ ; comparison of multiple groups:  $P = 0.0016$ ); B: Colony formation assay showed that IL-1RA single drug treatment decreased the colony formation of HCT116 cells, while this parameter significantly decreased in the HCT116 cells treated with 6.25 mg/mL 5-FU combined with 200 mg/mL IL-1RA compared with the untreated cells (5-FU vs Ctrl,  $P = 0.0104$ ; IL-1RA vs Ctrl,  $^aP = 0.0451$ ; 5-FU and IL-1RA vs IL-1RA,  $^bP = 0.0063$ ; comparison of multiple groups:  $P = 0.0013$ ).

which can promote the activity of the NF-κB pathway in carcinoma cells<sup>[30]</sup>. Currently, only four of the drugs used to treat colorectal cancer are related to genetic mutations,



**Figure 7 Interleukin-1RA enhances the chemosensitivity to fluorouracil and delays the survival time of tumor-bearing nude mice.** A and B: The tumor size of the control group treated with PBS (100  $\mu$ L/mouse) for 3 wk significantly increased compared to that in the fluorouracil (5-FU) group and the 5-FU and interleukin (IL)-1RA group (dose per nude mouse: 20 mg/kg of 5-FU; 1.5 mg of IL-1RA); C: The tumor weights (g) of the nude mice in the 5-FU and IL-1RA group were decreased compared with those in the 5-FU group (5-FU group vs Ctrl:  $^aP < 0.0001$ ; 5-FU and IL-1RA group vs 5-FU group:  $^bP = 0.0006$ ; comparison of multiple groups:  $P < 0.0001$ ); D: The tumor volumes ( $\text{cm}^3$ ) of nude mice in the 5-FU and IL-1RA group were decreased compared with those in the 5-FU group and the control group (18<sup>th</sup> day: 5-FU and IL-1RA group vs Ctrl:  $P = 0.0007$ ; 5-FU and IL-1RA group vs 5-FU group:  $P = 0.0192$ ; comparison of multiple groups:  $P = 0.0001$ ); E: The tumor diameter (cm) of the nude mice in the 5-FU and IL-1RA group was decreased compared with that in the 5-FU group and the control group (18<sup>th</sup> day: 5-FU and IL-1RA group vs Ctrl:  $P < 0.0001$ ; 5-FU and IL-1RA group vs 5-FU group:  $P = 0.0086$ ; comparison of multiple groups:  $P < 0.0001$ ); F: The survival time (d) of nude mice with xenograft tumors treated with 5-FU and IL-1RA was significantly longer than that of the nude mice with tumors treated with 5-FU (log-rank test,  $P = 0.0008$ ; 5-FU and IL-1RA group vs 5-FU group:  $P = 0.0413$ ).

and biomarkers must be detected. The detection-based biomarkers for treatment include EGFR, MSI-H/dMMR, BRAF + MEK, and NTRK fusion targets<sup>[31,32]</sup>. These patients must have wild-type KRAS/NRAS/BRAF and only left-sided tumors after gene testing. However, the 5-year survival rate of colorectal cancer is only 11%<sup>[33]</sup>. This study attempted to extend the detection of biomarkers to provide guidance for precise treatment of Kras mutant colon cancer, thus improving the patient survival rate.

IL-1 receptor antagonist targets IL-1 and competitively inhibits the activity of the NF- $\kappa$ B pathway in Kras mutant colorectal cancer, and it was combined with 5-FU as a neoadjuvant chemotherapy in this study. IL-1 $\alpha$  and IL-1 $\beta$ , two types of IL-1, were associated with the NF- $\kappa$ B pathway and highly expressed in Kras mutant colon cancer cell lines, which suggested that IL-1 was closely related to the NF- $\kappa$ B pathway in colon

carcinoma. The NF- $\kappa$ B pathway plays an essential role in the transcriptional regulation in response to various stimuli<sup>[30]</sup>. Consistent with other studies, high expression of phosphorylated P65 could activate downstream genes and promote the proliferation of colon cancer cell lines, such as COLO205, SW620, and HCT116<sup>[34]</sup>. This change persistently activated the NF- $\kappa$ B pathway, which was associated with the mutant *Kras* gene as an oncogene that promotes the proliferation of colon cancer cells<sup>[34]</sup>. IL-1 and other interleukins, such as IL-6, IL-8, IL-17, and IL-21, and TLR4 are also highly expressed in colon cancer cells and are associated with the NF- $\kappa$ B pathway in a feedback loop<sup>[34]</sup>. In *Kras* mutant colon cancer cells, the activation of P65 was inhibited by siRNA, and decreased expression of interleukins was detected. Among the interleukins, IL-1 $\alpha$  and IL-1 $\beta$  were sensitive to changes in the NF- $\kappa$ B pathway, and a significant decrease was found in the si-P65 colon cancer cell line.

Inhibition of the interleukins associated with the NF- $\kappa$ B pathway could reduce the proliferation of tumor cells and promote their apoptosis<sup>[35,36]</sup>. A previous study found that inhibition of IL-6 could decrease the growth of cancer cells and promote cellular apoptosis<sup>[36,37]</sup>. Furthermore, IL-1 receptor antagonist combined with 5-FU chemotherapeutic drugs may achieve enhanced effects compared to 5-FU alone in treating the colon cancer cell lines SW620 and HCT116. IL-1 RA could counteract the abnormally high expression of P-TAK1, P-P65, and P-MEK caused by 5-FU in the SW620 cell line. The anti-pyrimidine chemotherapeutic drug 5-FU is currently one of the most commonly used drugs in the clinic<sup>[38]</sup>. Clinical studies have shown that 5-FU has certain therapeutic effects on many kinds of tumors, such as digestive tract tumors, breast cancer, ovarian cancer, chorionic carcinoma, cervical cancer, liver cancer, bladder cancer, skin cancer (local smear), and leukoplakia (local smear), and inhibits the synthesis of DNA<sup>[39]</sup>. This drug has been clinically used in various chemotherapy regimens for colon cancer. Studies have shown that 5-FU has notable side effects, including bone marrow suppression, gastrointestinal reaction, and hair loss<sup>[40]</sup>. A previous study found that 5-FU chemotherapy combined with IL-6 inhibitors could better inhibit the growth of colon cancer cells than single treatment<sup>[41]</sup>, and a low dose of 5-FU combined with IL-6 inhibitors can achieve the same effect and reduce the side effects of chemotherapy<sup>[42]</sup>.

In this study, the combination treatment of 5-FU and IL-1 receptor antagonist in SW620 and HCT116 cell lines significantly inhibited the cell proliferation compared with single 5-FU treatment. The activation of TAK1 and high expression of MEK may lead to the proliferation of malignant cells with drug resistance to 5-FU. The chemosensitivity to 5-FU could be enhanced by IL-1 receptor antagonist, which inhibits the expression of phosphorylated P65, TAK1, and MEK in SW620 and HCT116 cell lines. In the clonogenicity assay, IL-1 receptor antagonist combined with 5-FU had a stronger effect in inhibiting cell clone formation than 5-FU alone. Therefore, treatment with IL-1 receptor antagonist combined with 5-FU can reduce the 5-FU dose to achieve an inhibitory effect on the proliferation of colon cancer cells and reduce the side effects of chemotherapeutic drugs. Notably, treatment with IL-1 receptor antagonist alone in this study did not achieve a significant inhibitory effect on the proliferation of *Kras* mutant colon cancer cells. A low dose of IL-1 receptor antagonist (50 mg/mL, 100 mg/mL) combined with 5-FU did not have a strong inhibitory effect on the proliferation of colon cancer cells.

In summary, the mutant *Kras* gene can promote the activity of the NF- $\kappa$ B pathway in colon carcinoma cells. There is a feedback loop between the upregulated NF- $\kappa$ B pathway and IL-1, which leads to the proliferation of cancer cells. The chemotherapy drug 5-FU can activate the NF- $\kappa$ B pathway and lead to chemotherapy resistance in colon carcinoma cells. IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than single drug treatment due to the blockade of IL-1. More experiments are needed to confirm and explore the underlying mechanism to provide a potential adjuvant chemotherapy for the clinic and a theoretical basis for neoadjuvant chemotherapy.

## ARTICLE HIGHLIGHTS

### Research background

The systemic toxicity and drug resistance of tumor cells are still two major problems in cancer chemotherapy. The chemotherapeutic drug 5-fluorouracil (5-FU) can inhibit the synthesis of adenylate synthetase and interfere with the synthesis of DNA in tumor cells. The effect of 5-FU is not ideal due to the chemoresistance in colon carcinoma patients treated with 5-FU.

### Research motivation

Interleukin (IL)-1 and nuclear factor kappa-B (NF- $\kappa$ B) show a cyclic relationship, which leads to persistent activation of NF- $\kappa$ B in tumor cells. In *Kras* and *p53* gene mutant mice, we found that the activity of NF- $\kappa$ B was downregulated by inhibiting the IL-1 receptor, which could effectively slow tumor growth.

### Research objectives

The aim of this study was to determine whether treatment with 5-FU combined with IL-1 receptor antagonist can increase the chemosensitivity to 5-FU by decreasing the activation of the NF- $\kappa$ B pathway and reducing the proliferation of colon cancer cells. The results obtained provide a theoretical basis for clinical adjuvant chemotherapy.

### Research methods

The expression of phosphorylated P65 in the COLO205, SW620, and HCT116 cell lines was significantly higher than that in the NCM460 cell line, as shown by Western blot assays. We used a xenograft nude mouse model to demonstrate the downregulation of the NF- $\kappa$ B pathway by blocking the NF- $\kappa$ B-regulated IL-1 $\alpha$  feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting colon tumor cell growth.

### Research results

IL-1RA combined with 5-FU showed stronger inhibition of the proliferation of the SW620 and HCT116 cell lines than 5-FU treatment. IL-1RA combined with 5-FU treatment had a greater effect in extending the survival time of the tumor-bearing nude mice than 5-FU single therapy.

### Research conclusions

IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than 5-FU alone due to the blockade of IL-1.

### Research perspectives

These results could provide an adjuvant chemotherapy strategy for the clinic and provide a theoretical basis for neoadjuvant chemotherapy.

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