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The effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways.

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Keywords: human colorectal cancer cells; HCT116 cells; growth cycle; proliferation; apoptosis.

Abstract. The purpose of this work was to investigate the effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways. Human colorectal cancer HCT116 cells were cultured and divided into a control group and low, medium and high-dose curcumin groups (n =5). HCT116 colorectal cancer cells became long-growing cells after incubation and culture at 37°C. The control group was treated with 15μL phosphate-buffered saline, and the low-dose, medium-dose and high-dose curcumin groups were treated with 20, 40 and 80μmol/L curcumin, respectively. All groups were treated with relevant drug intervention, digested and centrifuged for 48h, washed twice with a PBS solution, centrifuged at 1000 rpm for 3 min, and the cells precipitated. The proliferation, apoptosis and growth cycle of cells in each group were observed, and the expressions of the JAK/STAT3 and RAS/MAPK/NF-κB pathways and related proteins in each group were studied. Compared with the curcumin low-dose and medium-dose groups, the proliferation ability of the curcumin high-dose group was significantly decreased ($P<0.05$). When the low-dose and medium-dose curcumin groups were compared with the high-dose curcumin group, the apoptosis ability was significantly increased ($P<0.05$). When the low-dose and medium-dose curcumin groups were compared, the growth ratio of the G0/G1 phase in the high-dose curcumin group was significantly increased, and the percentage of the S phase was significantly decreased ($P<0.05$). Compared with the curcumin low-dose and medium-dose groups, the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway in the curcumin high-dose group was significantly decreased ($P<0.05$). The protein expressions of STAT3, RAS, P-P38 and P65 in the curcumin high-dose group were significantly lower than those in the curcumin low-dose and medium-dose groups ($P<0.05$). Curcumin can inhibit the expression of JAK/STAT3 and RAS/MAPK/NF-κB pathways, block the growth cycle, and inhibit the proliferation and induce apoptosis of colorectal cancer cells, providing a new idea for the clinical treatment of colorectal cancer.

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Los efectos de la curcumina en el comportamiento biológico de las células del cáncer colorrectal mediante las vías JAK/STAT3 y RAS/MAPK/NF-KB.

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Palabras clave: células de cáncer colorrectal humano; células HCT116; ciclo de crecimiento; proliferación; apoptosis.

Resumen. El objetivo del presente trabajo fue investigar los efectos de la curcumina en el comportamiento biológico de las células del cáncer colorrectal mediante el estudio de las vías JAK/STAT3 y RAS/MAPK/NF-KB. Las células del cáncer colorrectal humano HCT116 se cultivaron y dividieron en un grupo control y en grupos con dosis baja, media y alta (n = 5) de curcumina. Las células de cáncer colorrectal HCT116 se convirtieron en células de crecimiento prolongado después de la incubación y cultivo a 37°C. El grupo de control se trató con 15 μL de solución tampón fosfato salina (PBS) y los grupos de curcumina de dosis baja, media y alta se trataron con 20, 40 y 80 μmol/L de curcumina, respectivamente. Todos los grupos fueron tratados con la intervención farmacológica pertinente, digeridos y centrifugados durante 48 horas, lavados dos veces con solución de PBS, centrifugados a 1000 rpm durante 3 minutos, y las células precipitadas. Se observaron la proliferación, la apoptosis y el ciclo de crecimiento de las células de cada grupo, y fueron estudiados las expresiones de las vías JAK/STAT3, RAS/MAPK/NF-KB y proteínas relacionadas en cada grupo. Comparado con los grupos de la dosis baja y media de la curcumina, disminuyó obviamente la capacidad de proliferación del grupo de la dosis alta de la curcumina (P<0,05). Comparado con los grupos de la dosis baja y media de la curcumina, aumentó de modo significativo la capacidad de la apoptosis del grupo de la dosis alta de la curcumina (P<0,05). Comparado con los grupos de la curcumina de dosis baja y media, aumentó obviamente la proporción del crecimiento de la fase G0/G1 en el grupo de la curcumina de dosis alta y el porcentaje de la fase S disminuyó considerablemente (P<0,05). Las expresiones proteicas STAT3, RAS, P-P38 y P65 en el grupo de la dosis alta de la curcumina fueron evidentemente más bajas que las de los grupos de la dosis baja y media de la curcumina (P<0.05). La curcumina puede inhibir la expresión de las vías JAK/STAT3 y RAS/MAPK/NF-KB, bloquear el ciclo del crecimiento y luego inhibir la proliferación e inducir apoptosis de las células del cáncer colorrectal, lo que brinda una nueva idea para el tratamiento clínico del cáncer colorrectal.

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INTRODUCTION

Colorectal cancer is a common gastrointestinal malignancy in clinical practice, ranking third among malignancies worldwide, and its mortality rate is second only to liver cancer and lung cancer [1]. Data survey shows that the incidence and mortality of colorectal cancer are on the rise, and its mortality rate ranks second among malignant tumors in developed countries, seriously threatening human health and quality of life [2]. Studies have

found that the incidence of colorectal cancer is gradually rising in younger people, and the prognosis of patients underyears old-old is poor [3]. Surgical resection is currently the primary method treatingt for colorectal cancer in clinical practice. Patients in advanced stages are mostly treated with radiotherapy and chemotherapy, but the treatment effect of colorectal cancer is not ideal; and the prognosis of the patients is poor, and most patients suffer from recurrence and metastasis [4]. Curcumin is a natural and effective chemical component in plants. It mainly exists in the roots and stems of turm,eric and it is insoluble in water. It can change with the change of acid and alkali conditions. Curcumin is widely used in food production as a preservative, colorant, etc., and also plan essentialtant role in anti-tumor, hypolipidemic, anti-oxidation and anti-arteriosclerosis[5]. Jabus kinases/signal and activator of the transcription Jak-stat pathway is involved in the cycle, cell transformation and apoptosis of tumor cells [6]. It was found [7] that Rat sarcoma (RAS) protein can activate the mitogen activated protein kinase (MAPK) and the nuclear factor (NF-κB) pathway. It can induce the proliferation of hepatocellular carcinoma cells and strengthen their invasion ability. However, curcumin has been rarely studied in the JAK/STAT3 and RAS/MAPK/NF-κB pathways of colorectal cancer. Therefore, this study aimed to explore the effects of curcumin on the biological behavior of colorectal cancer cells through the JAK/STAT3 and RAS/MAPK/NF-κB pathways.

MATERIAL AND METHODS

Experimental materials

Human colorectal cancer HCT116 cells were purchased from Hubei Punosai Life Science and Technology Co., LTD., and were uniformly cryopreserved by the experimental center of our hospital.

Experimental instruments and reagents

Curcumin (Beijing Green Heng Xing Biotechnology Co., LTD.), PBS buffer (Beijing Standard Technology Effective Company), medium (Shanghai Biotechnology Co., LTD.), fetal calf serum (Jiangsu Ke Wei Biotechnology Co., LTD.), dimethyl sulfoxide (Chengdu Medical Technology Co., LTD.), automatic labeling instrument (Shanghai Molecular Instrument Co., LTD.,), protein extraction kit (Beijing Solaibao Technology Co., LTD.), flow cytometer (Shanghai Huanlian Medical Device Co., Ltd.).

Cell Grouping

Colorectal cancer HCT116 cells were placed in 10% fetal bovine serum and cultured in a cell incubator with 5% CO_2 and 37°C. When the cells were fused to 90%, a trypsin solution was given for digestion and passage treatment. When the passage reached the third generation, cells with long-term growth were selected for experiment. In the process of culture, the growth state of cells was observed, and the conventional fluid was changed according to its state. Colorectal cancer HCT116 cells were divided into control group, and a curcumin low-dose, medium-dose and high-dose groups. The control group was treated with 15μL phosphate buffered saline, and the low-dose, medium-dose and high-dose curcumin groups were treated with 20, 40 and 80 μmol/L curcumin, respectively, to observe the proliferation and apoptosis of cells in each group. The expression of JAK/STAT3, RAS/MAPK/NF-κB pathway and related proteins were studied in each group.

EXPERIMENTAL METHODS

Cell proliferation experiment

The cell cycle is the most important entity for cell survival. Abundant factors and proteins in positive or negative maps at multiple points and bottlenecks, precisely and harmoniously regulate and control this cycle. In fact, there are a variety of genes in cells that encode proteins needed to control the cell cycle. Although the cell cycle is monitored and inspected at several stations,

this adjustment takes place especially at two points with extraordinary intensity and care. The cell, first, decides to replicate its own DNA, and second, to initiate mitotic division. These steps are in the realm of passing from G1 to S and from G1 to M.

The cells in each group were successively inoculated into a 96-well culture plate, and the cell density was adjusted to 10×10^4/mL. The cells were observed for 5h, and the corresponding intervention was given to each group. Cultured again for 36 h, the MTT assay was used to observe the situation of each group of cells, and the proliferation of each group was plotted. The experiment was repeated 3 times.

Flow cytometry detection

Colorectal cancer HCT116 cells with a concentration of 2×10^8/L were inoculated into 6-well culture plates for 12 h. The control group was given routine culture, and the curcumin low-dose, medium-dose and high-dose groups were given 20, 40 and 80 μmol/L curcumin for intervention. After 48 h of intervention, the cells were collected and centrifuged at 1000 rpm for 10 min. The cells were washed with PBS twice, and 1ml of precooled 70% ethanol was used for beating and dispersing. The cells were fixed overnight at -20°C, and then mixed with 0.8 μg/mL. The cells were incubated at room temperature for 30 min without light.

Western blot assay

Western blot analysis was performed essentially according to standard protocol. Briefly, the cells were solubilized in lysis buffer (50 mM Tris, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 2.5 mM Na3O4V, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 25 μg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). After clarification at 10,000g for 15 minutes, the supernatant was used for Western blot analysis. In all analyses, protein concentration, determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), was standardized among the samples. Aliquots of cell lysates containing 50 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred electrophoretically onto supported nitrocellulose membranes (Osmonics, Gloucester, MA). Membranes were incubated for 1 hour at room temperature with blocking buffer, TBS-T (20 mM Tris, pH7.6, 100 μMNaCl, 0.1% Tween-20) and 5% nonfat dry milk with gentle agitation. After washing the membranes with TBS-T, they were incubated overnight at 4°C in TBS-T buffer containing antibody dilution buffer as suggested by the manufacturer and with antibodies (1:1000 dilution) to CD44, CD166 (Santa Cruz Biotechnology, Santa Cruz, CA), or epidermal growth factor receptor (EGFR; Cell Signaling, Beverly, MA). The membranes were washed three times with TBS-T and subsequently incubated with appropriate secondary antibodies (1:5000 dilutions) in TBS-T containing 5% milk for 1 to 2 hours at room temperature with gentle agitation. The membranes were washed again with TBS-T, and the protein bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ). The membranes containing the electrophoresed proteins were exposed to X-Omat film (Sigma-Aldrich, St Louis, MO). The membranes were stripped (twice × for 15 minutes at 55°C) in stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl pH 6.7, and reprobed for β-actin using the corresponding antibodies, which were used as a loading control. All Western blots were performed at least three times for each experiment [8]. The method used to identify proteins in the membrane is the blotting method. In this technique, protein bands are transferred from the gel to a nitrocellulose membrane that can bind and stabilize proteins. To do this, by blotting, the protein molecules were removed from the gel and placed in the same position on the surface of the membrane, so we could easily study them, separate

them, and finally use them. Specific ligands or substrates were used to detect proteins or enzymes transferred to the membrane. Antibodies were also used to specifically detect proteins in the membrane.

All groups were treated with relevant drug intervention, digested and centrifuged for 48h, washed twice with PBS solution, centrifuged at 1000 rpm for 3min, and the cells precipitated. The protein was extracted, 200μL lysate was put into the cell sample, let stand and mix. The BCA method was used to determine protein content according to the kit instructions. After gel preparation, sample loading, electrophoresis, membrane transfer, elution, sealing, primary antibody incubation, membrane washing, secondary antibody incubation and membrane washing again, the protein expression was calculated by the Invitrogen™ /Thermo Fischer Scientific, USA, technique.

Apoptosis methodology

There are two pathways for cell apoptosis, intrinsic and external. For this study, the TUNEL method was used to evaluate apoptosis [9].

To diagnose apoptosis, the TUNEL kit (insitu cell death detection kit, POD, Roche company, made in Germany) (Cat. No. 11 684 817 910) was used. The TUNEL technique was performed as follows:

1. First, the prepared sections were washed with para-proteinase K after paraffinization and irrigation, and after incubation, they were washed with buffer phosphate solution for 30 minutes at 37°C.

2. Tissue sections were stained with 50 μl of TUNEL reaction solution for 37 minutes at 37°C, then washed with buffer phosphate solution.

3. At this stage, the sections were then washed with a converter solution (50 μL) for 30 minutes at 37°C with buffer phosphate solution and then mixed with a diamino benzidine solution for 20 minutes and then stirred for 25 minutes. They were incubated again at 37°C.

4. The sections were then washed three times with phosphate solution and batoloidin blue, for two minutes each time.

Cells were manually examined in 10 random high-power (×100 magnification) fields (>1000 cells) and the apoptosis index (AI) was determined as follows: AI = number of positively stained cells/total number of cells counted.

Statistical methods

The SPSS20.0 software package was used for statistical analysis of the study data, and the measurement data was expressed as means ± standard deviation ($\bar{x} \pm s$). One way-ANOVA was used for comparison between groups. $P<0.05$ was taken as the statistical standard.

RESULTS

Comparison of proliferation ability of different groups of cells

Compared with the control group, the proliferation ability of the medication group was significantly decreased, with statistical significance ($P<0.05$); compared with the low-dose and medium-dose curcumin groups, the proliferation ability of the high-dose curcumin group was significantly decreased, with statistical significance ($P<0.05$), as shown in Table 1.

Comparison of apoptosis in different groups

Compared with the control group, the apoptotic ability of the medication group was significantly increased ($P<0.05$); compared with the low-dose and medium-dose curcumin groups, the apoptotic ability of the high-dose curcumin group was significantly increased ($P<0.05$), as shown in Table 2.

Comparison of cell growth cycles of different groups

Compared with the control group, there was no significant difference in G2/M phase ($P>0.05$), but the growth ratio of G0/

Table 1
Comparison of migration ability (proliferation) of different groups of cells ($\bar{x} \pm s$)

Group	Number of stems	proliferation ability（%）
control group	5	96.44 ± 3.15
low-dose curcumin group	5	76.16 ± 5.33*
medium-dose curcumin group	5	59.13 ± 4.16*#
high-dose curcumin group	5	45.11 ± 3.85*#△
F		139.29
P		＜0.001

Compared with control group, *P<0.05; compared with curcumin low-dose group, #P<0.05; compared with curcumin medium-dose group, △P<0.05. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

Table 2
Comparison of apoptosis in different groups ($\bar{x} \pm s$)

Group	Number of stems	Apoptosis (%)
control group	5	5.23 ± 1.32
low-dose curcumin group	5	14.35 ± 1.22*
medium-dose curcumin group	5	25.76 ± 1.68*#
high-dose curcumin group	5	35.79 ± 2.11*#△
F		337.77
P		<0.001

Compared with control group, *p < 0.05; compared with curcumin low-dose group, #p< 0.05; compared with curcumin medium-dose group, △p< 0.05. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

G1 phase in the treatment group was significantly increased, and the percentage of S phase was significantly decreased, the difference was statistically significant (P<0.05). Compared with the low-dose and medium-dose curcumin groups, the growth ratio of G0/G1 phase in the high-dose curcumin group was significantly increased. The percentage of S stage decreased significantly, with statistically significant difference (P<0.05), as shown in Table 3.

Comparison of JAK-STAT3 and RAS/MAPK/NF-κB pathway expression in different groups

Compared with the control group, the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway in the treatment group was significantly decreased, and the differences were statistically significant (P<0.05).The expression of JAK-STAT3 and RAS/MAPK/NF-κB pathway (μg) was significantly decreased in the high-dose curcumin group, and the differences were statistically significant (P<0.05), as shown in Table 4.

Comparison of related protein expression in different groups

Compared with the control group, the protein expressions (μg) of STAT3, RAS, P-P38 and P65 in the medication group were significantly decreased, with statistical significance (P<0.05). Compared with the low-dose and medium-dose curcumin groups, the protein expressions of STAT3, RAS, P-p38 and P65 in the high-dose curcumin group were significantly decreased. The difference was statistically significant (P<0.05), as shown in Table 5.

Table 3
Comparison of cell growth cycles of different groups ($\bar{x} \pm s$)

Group	Number of stems	G0/G1(%)	S(%)	G2/M(%)
control group	5	33.26 ± 2.19	53.76 ± 3.11	11.52 ± 3.16
low-dose curcumin group	5	45.38 ± 2.56*	41.35 ± 2.94*	11.31 ± 2.25
medium-dose curcumin group	5	59.67 ± 3.12*#	30.85 ± 3.16*#	10.23 ± 1.35
high-dose curcumin group	5	71.31 ± 3.15*#△	20.33 ± 2.08*#△	9.22 ± 1.64
F		177.61	125.62	1.00
P		<0.001	<0.001	0.418

Compared with control group, *$p<0.05$; compared with curcumin low-dose group, #$p<0.05$; compared with curcumin medium-dose group, △$p<0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells.

Table 4
Comparison of JAK-STAT3 and RAS/MAPK/NF-κB pathway expression (μg) in different groups ($\bar{x} \pm s$)

Group	Number of stems	JAK-STAT3	RAS	p38MAPK	NF-κB
control group	5	1.06±0.09	1.15±0.26	0.98±0.02	0.85±0.16
low-dose curcumin group	5	0.71±0.11*	0.73±0.09*	0.75±0.11*	0.54±0.05*
medium-dose curcumin group	5	0.51±0.06*#	0.53±0.05*#	0.42±0.07*#	0.40±0.05*#
high-dose curcumin group	5	0.31±0.02*#△	0.29±0.04*#△	0.26±0.03*#△	0.22±0.04*#△
F		32.07	33.24	114.71	43.99
P		<0.001	<0.001	<0.001	<0.001

Compared with control group, *$p<0.05$; compared with curcumin low-dose group, #$p<0.05$; Compared with curcumin medium-dose group, △$p<0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells. Unit of protein values: μg.

Table 5
Comparison of related protein expression (μg) in different groups ($\bar{x} \pm s$)

Group	Number of stems	STAT3	ras	p-p38	p65
control group	5	1.31 ± 0.35	1.53 ± 0.41	1.35 ± 0.31	1.45 ± 0.15
low-dose curcumin group	5	0.92 ± 0.19*	1.02 ± 0.19*	0.85 ± 0.08*	0.73 ± 0.11*
medium-dose curcumin group	5	0.59 ± 0.06*#	0.62 ± 0.08*#	0.51 ± 0.06*#	0.52 ± 0.05*#
high-dose curcumin group	5	0.29 ± 0.02*#△	0.28 ± 0.01*#△	0.26 ± 0.01*#△	0.21 ± 0.03*#△
F		23.64	27.48	41.90	146.12
P		<0.001	<0.001	<0.001	<0.001

Compared with control group, *$p < 0.05$; compared with curcumin low-dose group, #$p< 0.05$; compared with curcumin medium-dose group, △$p< 0.05$. P-Value based on One Way- ANOVA. Number of stems: number of groups of cells. Unit of protein values: μg.

DISCUSSION

With the changes of diet structure and the improvement of living standards, the incidence of colorectal cancer has been high, and is closely related to genetics, the environment and diet. Data survey shows that the incidence of colorectal cancer in big cities in China is higher than that in other cities, and it shows an increasing trend year by year. Colorectal cancer has become the most common and fastest growing malignant tumor in China [10]. Surgery combined with radiotherapy and chemotherapy is commonly used in the clinical treatment of colorectal cancer, but about 50% of patients will have recurrence and metastasis after surgery, which will eventually lead to death of patients [11]. The application of chemotherapy drugs can lead to serious toxic and side effects in patients, waste medical resources, and bring serious economic burden to families and society. Therefore, finding effective and safe drugs to treat colorectal cancer has become the focus of clinical research.

Traditional Chinese medicine treatment has been gradually applied in clinical practice, and has unique advantages, and has become an important part of clinical tumor treatment. Studies have found [12] that curcumin widely exists in turmeric, which can be combined with chemoradiotherapy drugs to effectively reduce the toxic and side effects of chemotherapy drugs, the dosage of these drugs, and to improve the prognosis and the quality of life of patients. Curcumin has been applied to thyroid cancer cells, and inhibition of thyroid cancer cell proliferation was observed, which may be related to the inhibition of p-MTOR and P-S6K proteins in thyroid cancer [13]. Another study in thyroid cancer found that curcumin could down-regulate the expressions of cy-Clinbl and Bcl-XL, and then inhibit thyroid cancer cells, thereby promoting their apoptosis [14]. The results were similar to those of this group. The cell growth cycle is the most important step in tumorigenesis and development. Foreign studies have found that when curcumin is applied to colorectal cancer cells, it is found that G1 block occurs, and then the apoptosis specific DNA is delayed, suggesting that curcumin can regulate the growth cycle of colorectal cancer cells [15]. In this experiment, compared with curcumin low-dose and medium-dose groups, the proliferation ability of curcumin high-dose group was significantly decreased, and the apoptosis ability was significantly increased ($P<0.05$). These results suggest that curcumin can inhibit the proliferation of colorectal cancer cells, block the growth of colorectal cell cycle, and induce their apoptosis.

During the occurrence and development of gastric cancer, the Jak-Stat3 pathway and the RAS-MAPK pathway have been successively activated and have complex interactions, which are closely related to gastric cancer. MAPK is a core member of the RAS-MAPK pathway, which can transmit extracellular signals to the nucleus by activating MAPK, leading to activation of phosphoamino acid residues of Jun, FOS and other transcription factors in the nucleus, thus regulating gene expression and finally leading to cell growth and differentiation [16]. The terminal of STAT3-C has a serine residue, and Ser727 is the phosphorylation site of MAPK, indicating that STAT3 is closely related to p38MAPK. Studies have confirmed that STAT3 has a certain correlation with p38MAPK in gastric cancer, and the expression of STAT3 can affect the expression of p38MAPK, which increases with the increase of STAT3 [17]. RAS exists in the form of binding proteins in DNA and mutates in tumors, thus affecting the protein activity of RAS and accelerating the proliferation, migration and invasion of tumors, and has become an important target of cancer [18]. It was found that MAPK/NF-κB can act as a downstream pathway of RAS, leading to RAS activation, which in turn activates MAPK enzyme, resulting in transfer and phosphorylation of Raf molecules downstream of RAS, thereby activating MAPK, and activating NF-κB in the nucleus, amplifying

RAS activity. Ultimately, the inflammatory response is accelerated [19]. In this study, the expressions of JAK-STAT3 and RAS/MAPK/NF-κB pathways were significantly decreased in the curcumin high-dose group, and the protein expressions of STAT3, RAS, P-P38 and P65 were significantly decreased in curcumin low-dose and medium-dose groups ($P < 0.05$). These results suggest that curcumin can inhibit the expression of JAK-STAT3 and RAS/MAPK/NF-κB pathways, further inhibit related proteins, and prevent further proliferation of colorectal cancer cells.

In this experimental study, curcumin could inhibit the proliferation and induce apoptosis of colorectal cancer cells by inhibiting the expression of JAK/STAT3 and RAS/MAPK/NF-κB pathways and block the growth cycle, providing a new idea for clinical treatment of colorectal cancer.

Conflict of interest

The manuscript has no conflict of interest.

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Limitation

Low number of groups of cells.

Authors Contribution

ZY and RZhao collected the samples. ZY and WG analysed the data. RZ and WG conducted the experiments and analyzed the results. All authors discussed the results and wrote the manuscript.

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