

ORIGINAL ARTICLE

Effect of PD98059 on chemotherapy in patients with colorectal cancer through ERK1/2 pathway

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Summary

Purpose: To investigate the effects of MAPK/ERK signaling transduction pathway inhibitor PD98059 combined with paclitaxel on the proliferative, invasive and apoptotic abilities of colorectal cancer cells.

Methods: Colorectal cancer (CRC) SW-480 cells were selected and divided into 3 groups after passage: Blank group (not treated), control group (treated with PD98059 blocker (25 μ mol/L)), observation group (treated with PD98059 blocker (25 μ mol/L) combined with paclitaxel (100 μ mol/L)).

Results: The proliferation, invasion and apoptosis of cells in each group were registered. The relative expressions of ERK1/2 and p-ERK1/2 protein were assessed by Western blot. The relative expressions of Bax and Bcl-2 were assessed by RT-PCR. The proliferation ability in the control group was significantly higher than that in the observation group ($p < 0.05$). The relative expression of p-ERK1/2 protein in the control group was significantly higher than that in the observation group ($p < 0.05$). The invasive ability in the blank group was significantly higher than that in the other two

groups ($p < 0.05$), and that in the control group was significantly higher than that in the observation group ($p < 0.05$). The apoptotic ability in the control group was significantly lower than that in the observation group ($p < 0.05$). The expression of Bcl-2 in the cells of the observation group was significantly higher than that of the blank group and the control group, and that in the control group was higher than that in the blank group ($p < 0.05$). The expression of Bax in the cells of the observation group was significantly lower than that of the blank group and the control group ($p < 0.05$).

Conclusion: Inhibitor PD98059 combined with paclitaxel can affect the expression of ERK1/2 in ERK1/2 signaling pathway, effectively inhibit the proliferation and invasive abilities of CRC cells, increase the apoptotic ability of CRC cells, and is expected to become a potential drug for clinical treatment of CRC.

Key words: PD98059, ERK1/2, colorectal cancer, proliferation, invasion

Introduction

Colorectal cancer (CRC) is a common malignant tumor of the digestive tract, ranking 4th in incidence in developing countries [1]. With the development of society, the living standards of residents have gradually increased, leading to an increase in the incidence of CRC and with an increasing trend year by year [2,3]. At present, the pathogenesis of CRC is not clear, but some studies [4] show that the occurrence of CRC is affected by environmental and

genetic factors, and more than 80% of CRCs derive from colorectal adenoma. Since there are no obvious clinical features and no significant effect on the daily life of the patient in the early stage of the disease, the patient is basically in the middle stage at the time of diagnosis, which leads to missing the best treatment opportunity.

Surgery is the routine way to treat CRC at present, and most of the patients can get marked

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improvement after surgery. However, it can only be performed to resect lesions for early cancer patients, and late CRC patients can only be treated by radiotherapy or chemotherapy [5,6]. Some authors [7] have shown that combination chemotherapy can effectively improve the survival of patients after surgery [7]. Paclitaxel, as a most widely used chemotherapeutic drug in the treatment of cancer, has obvious therapeutic effect on many kinds of tumors, such as lung cancer, ovarian cancer, CRC etc [8]. Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase family, regulates cell growth, development and division through signaling transduction pathways, and is often used as an important pathway for studying tumors [9]. A study has shown [10] that the inhibition of ERK1/2 pathway has an inhibitory effect on tumorigenesis and disease development. PD98059, as a specific small molecular inhibitor of ERK1/2, can act by inhibiting ERK1/2 to block the ERK pathway [11].

Therefore, the inhibition of ERK1/2 pathway by PD98059 and the effect of PD98059 combined with paclitaxel on the cancer cells of CRC patients were assessed to find a new treatment method for clinicians.

Methods

Main materials

CRC cell line SW-480 (ATCC cell bank, USA, CCL-228); RPMI-1640 culture medium; fetal bovine serum (FBS); trypsin (Gibco company, USA, 61870044, 10437028, 15400054); Annexin V-PE Cell apoptosis detection kit; CCK-8 Kit (Beyotime Biological Co., Ltd., Shanghai, China, C1065L, C0037); PD98059 blocker (Apexbio, USA, A1663); Paclitaxel liposome for injection (Nanjing Sike Pharmaceutical Co., Ltd., SFDA Approval No. H20030357); Transwell chamber; Matrigel glue (Thermo Scientific Inc., USA, A1142801, A1569601); Rabbit anti-ERK1/2 monoclonal antibody; rabbit anti-p-ERK1/2 monoclonal antibody; horseradish peroxidase-labelled sheep anti-rabbit IgG (CST Inc., USA, 8544, 4370, 7072); PCR instrument (ABI, USA, 7500). Bax, Bcl-2 primers were designed and synthesized by Shanghai Sangon Biotechnology Co., Ltd (Table 1). EasyPure miRNA Kit, TransScript II Two-Step RT-PCR SuperMix (Beijing Trans Gene Biotech, ER601-01, AH401-01).

Cell culture

SW-480 CRC cells were cultured in RPMI-1640 medium (containing 10% FBS, penicillin-streptomycin) and placed in a constant temperature incubator (humidity 95%, temperature 37°C, CO₂ 5%), 1-2d for one change of liquid. The digestion was carried out with 0.25% trypsin, and the experiment was passaged to the 1st or 2nd generation. Blank group, control group, observation group were established, in which the blank group cells were not treated, the control group cells were treated with PD98059 blocker (25µmol/L), and the observation group cells were treated with PD98059 blocker (25µmol/L) combined with paclitaxel (100µmol/L).

Cell proliferation assay

CRC cells in logarithmic growth phase were digested with 0.25% trypsin and prepared to suspension with RPMI-1640 culture medium (10% BFS), and then inoculated in a 96-well plate at a cell concentration of $1 \times 10^7/L$, adding 200µL per well. The cell growth was observed. After the cells confluence reached 70-80%, the PRMI-1640 culture medium (without any reagents) was changed and the cells were cultured for 24h, and each group was added with relevant reagents and continued to be cultured for 12h, 24h, 48h, 72h. Cell proliferation was measured at different time points in each group by CCK-8 kit, and growth curves were drawn. CCK-8 steps: 10µL of CCK-8 reagent was added to each well and cultured in a constant temperature incubator (humidity 95%, temperature 37°C, CO₂ 5%) for 4h. The optical density (OD) value was measured and observed at 490nm by microplate reader. Six repetition wells were set up and the experiment was repeated 3 times in each group, and the average value was taken for analysis.

Detection of protein relative expression by Western blot

CRC cells in logarithmic growth phase were digested with 0.25% trypsin and prepared to suspension with RPMI-1640 culture medium (10% FBS), and then inoculated into a 96-well plate at a cell concentration of $1 \times 10^7/L$, adding 200µL per well. The cell growth was observed. After the cells reached 70-80% confluence, the PRMI-1640 culture medium (without any reagents) was changed and the cells were cultured for 24h. The cells were collected, and then PMSF inhibitor and RIPA cleavage solution were added for protein cleavage, and centrifugation was performed after cleavage. The concentration of protein was detected by BCA kit, and the protein loading buffer was added for denaturation at 100°C for 5min and transferred to -20°C for storage. The protein was separated by 12% SDS-PAGE gel electrophoresis and

Table 1. Primer sequence

Gene		
Bax	5'-CACAACCTCAGCGCAAACATT-3'	5'-ACAGCCATCTCTCTCCATGC-3'
Bcl-2	5'-GAAGCACAGATGGTTGATGG-3'	5'-CAGCCTCAAGGTTCCAAT-3'
GAPDH	5'-CCACCCATGGCAAATTCATGGCA-3'	5'-TCAAGACGGCAGGTCAGGTCCACC-3'

transferred to PVDF membrane at 350mA. Five percent nonfat dry milk-TBST was used to block the non-specific antigen for 2h, and the rabbit anti-monoclonal antibody ERK1/2, p-ERK1/2 (1:500) was added and incubated overnight at 4°C. Cells were rinsed 3 times with TBST, 10 min/time. The cells were added with horseradish peroxidase-labelled sheep anti-rabbit second antibody IgG (1:10000) and incubated at room temperature for 2h, and then washed, developed, scanned, and imaged again with TBST. GAPDH was used as the internal control to calculate the relative expression of protein in this study. The experiments were performed in triplicate.

Cell invasion test by Transwell assay

The serum free PRMI-1640 medium and Matrigel matrix glue were used for dilution (1:5). The surface of the upper insert of Transwell was coated with 100µL dilute solution and dried in 37°C constant temperature incubator overnight. The cancer cells were cultured for 24h in PRMI-1640 medium (10% FBS) with different reagents, then the cells in logarithmic growth phase were digested with 0.25% trypsin, resuspended to serum-free cell suspension and counted. Each upper chamber of Transwell inserts was inoculated with 200µL suspension (2.5×10^5 /mL cells), and the lower chamber was added with 500µL PRMI-1640 medium (20% FBS), and then cultured at 37°C constant temperature incubator for 24h.

After culture, the inserts were removed and washed with PBS buffer solution, and the cells were treated with formaldehyde (100%), and were washed with PBS again. After drying, the cell membrane penetration was observed and counted. The experiments were performed in triplicate.

Flow cytometry

CRC cells in logarithmic growth phase were digested with 0.25% trypsin and prepared to suspension with RPMI-1640 culture medium (10% FBS), and then inoculated into a 96-well plate at a cell concentration of 1×10^7 /L, adding 200µL per well. The cell growth was observed. After the cells' confluence reached 70-80%, the PRMI-1640 culture medium (without any reagents) was changed and the cells were cultured for 24h, and each group was added with relevant reagents and continued to culture for 12h, 24h, 48h, 72h. The three groups of cells in the culture plate were transferred to the cone-shaped tube and placed on the ice surface. The cells were washed with PBS solution and digested with 0.25% trypsin, tapping the culture plate to make the complete detachment of cells. The cells were resuspended in buffer solution (1×10^6 /mL) and transferred to the centrifuge tube. Operation was performed by AnnexinV-FITC kit and flow cytometry (Beckman-CytoFLEX) was used to analyze the apoptosis for a total of 3 times.

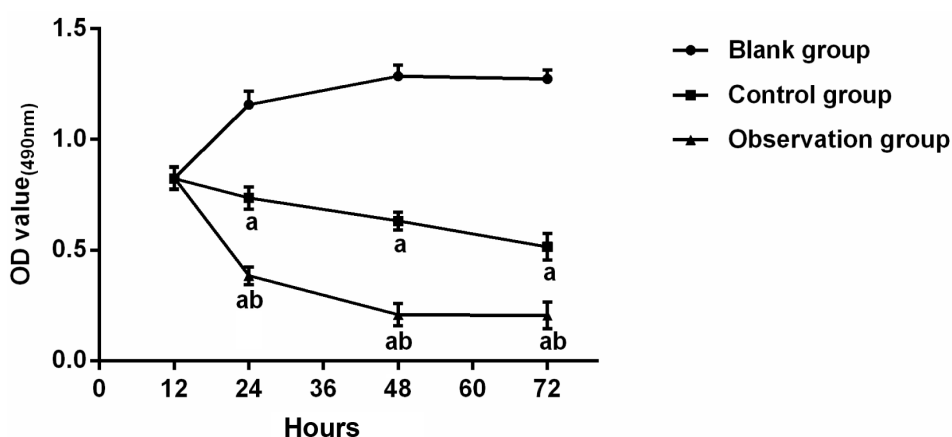


Figure 1. Cell proliferation. Detection of the cell proliferation by CCK-8 found that the proliferation ability in the blank group was significantly increased compared with the other two groups after 24 h, while the proliferation ability in the control group treated with PD98059 was inhibited, but still significantly higher than that in the observation group. "a" shows that there is a difference compared with the blank group ($p < 0.05$), and "b" shows that there is a difference compared with the control group ($p < 0.05$).

Table 2. Cell proliferation

Time	Blank group	Control group	Observation group	F	p
12h	0.825±0.05	0.823±0.05	0.827±0.05	0.010	0.991
24h	1.157±0.06	0.735±0.05 ^a	0.384±0.04 ^{ab}	350.187	0.000
48h	1.286±0.05	0.632±0.04 ^a	0.208±0.05 ^{ab}	804.353	0.000
72h	1.273±0.04	0.515±0.06 ^a	0.205±0.06 ^{ab}	617.485	0.000

"a" indicates that there is a difference compared with the blank group ($p < 0.05$), and "b" indicates that there is a difference compared with the control group ($p < 0.05$).

PCR detection

Total RNA was extracted from the collected cells by EasyPure miRNA Kit, and the purity, concentration and integrity of the extracted total RNA were determined by UV spectrophotometer and agarose gel electrophoresis. 5×TransScript® All-in-One SuperMix for PCR was used for reverse transcription of total RNA, and the procedure was performed in strict accordance with the manufacturer's instructions. According to TransScript II Two-Step RT-PCR SuperMix, 7500PCR instrument was used for the PCR amplification experiment. PCR reaction system: cDNA 2μL, upstream primer 1μL, downstream primer 1μL, 2×TransTaq® HiFi PCR SuperMix II 25μL, and Nuclease-free was complemented to 50μL. PCR reaction conditions: 40 cycles of pre-denaturation at 94°C for 5min, denaturation at 94°C for 50s, annealing at 60°C for 30s, elongation at 72°C for 5min. GAPDH was used as an internal parameter and $2^{-\Delta\Delta ct}$ was used to analyze the data in this study.

Statistics

SPSS 20.0 software package (Guangzhou Pomine) was used for the statistical analyses of the collected data in this study. GraphPad Prism 7 (Shanghai Becca) was used to draw the figures. The measurement data were expressed in the form of mean ± standard deviation (Meas±SD). One way analysis of variance (ANOVA) was used for multigroup comparisons, and comparison of different time points was performed by repeated measurements of ANOVA represented by F. The differences in the groups were analyzed by LSD-t test. $P < 0.05$ showed that there was statistical difference between the groups.

Results

Cell proliferation

OD value of cells in each group after intervention found that there was significant difference

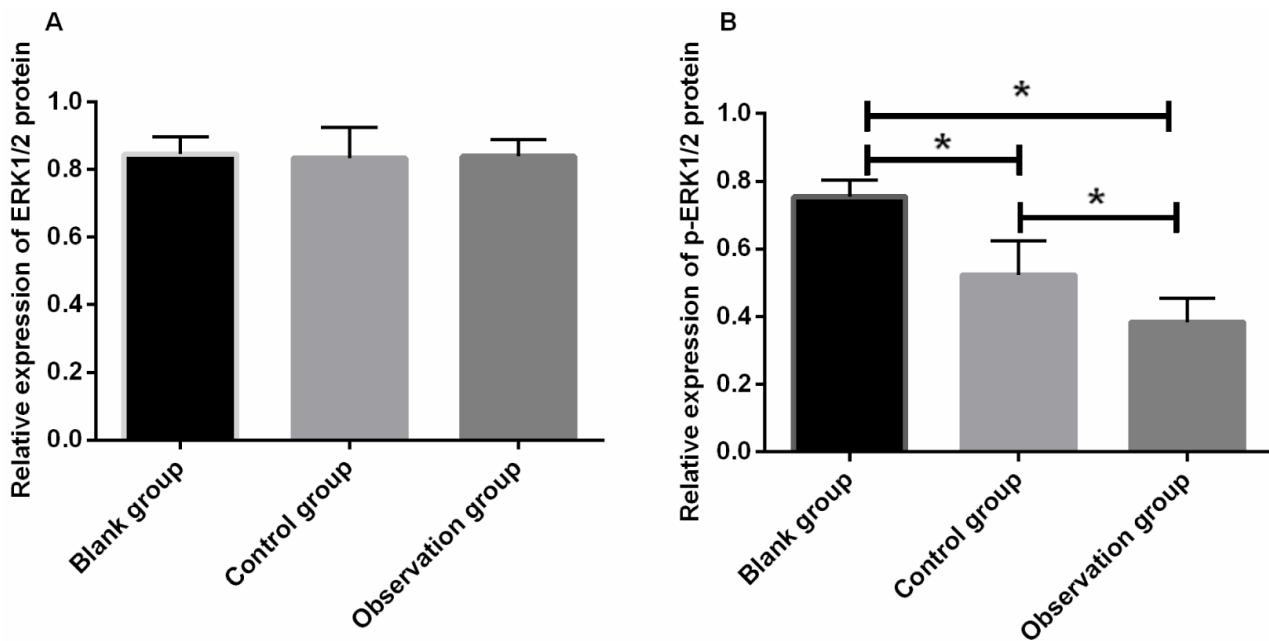


Figure 2. Expression of ERK1/2 protein in cells. **A:** Western blot analysis showed that there was no difference in the relative expression of ERK1/2 protein in each group ($*p > 0.05$). **B:** Western blot analysis showed that the relative expression of p-ERK1/2 protein in the blank group was significantly higher than that in the other two groups ($*p < 0.05$), and the relative expression of p-ERK1/2 protein in the control group was significantly higher than that in the observation group, with a statistical difference ($*p < 0.05$).

Table 3. Expressions of ERK, p-ERK protein in cells

Group	ERK1/2	F	p value	p-ERK1/2	F	p value
Blank group	0.845±0.05			0.754±0.05		
Control group	0.834±0.09 ^a	0.021	0.979	0.524±0.10	18.052	0.003
Observation group	0.839±0.05 ^{ab}			0.384±0.07		

"a" shows that there is a difference compared with the blank group ($p < 0.05$) and "b" shows that there is a difference compared with the control group ($p < 0.05$).

in the proliferation between blank group, control group and observation group after 24 hours ($p < 0.05$). The proliferation ability in the blank group was significantly faster than that in the control group and the observation group with a significant difference ($p < 0.05$), and the proliferation ability in the control group was significantly higher than that in the observation group with a statistical difference ($p < 0.05$) (Figure 1, Table 2).

Expressions of ERK, p-ERK protein in cells

Detection of the relative expressions of ERK1/2, p-ERK1/2 protein by Western blot assay showed that there was a significant difference in the relative expression of p-ERK1/2 protein in each group ($F_{p-ERK} = 18.052$, $P_{p-ERK} = 0.003$), and there was no difference in the relative expression of ERK1/2 protein in each group ($F_{ERK} = 0.021$, $P_{ERK} = 0.979$). The relative expression of p-ERK1/2 protein in the blank group was significantly higher than that in the other two groups with a statistical difference ($p < 0.05$), and the relative expression of p-ERK1/2 protein in the control group was significantly higher than that in the observation group with a statistical difference ($p < 0.05$) (Figure 2, Table 3).

Cell invasion

Detection of cell invasion by Transwell inserts found that there was a significant difference in cell invasive ability between blank group, control group and observation group ($F = 32.612$, $p = 0.001$). The invasive ability in the blank group was significantly higher than that in the other two groups with a statistical difference ($p < 0.05$), and that in the control group was significantly higher than that in

the observation group with a statistical difference ($p < 0.05$) (Table 4).

Cell apoptosis

Detection of cell apoptosis by flow cytometry found that there was a significant difference in apoptosis between blank group, control group and observation group ($F = 62.229$, $p = 0.000$). The apoptotic ability in the blank group was significantly lower than that in the other two groups with a statistical difference ($p < 0.05$), and that in the control group

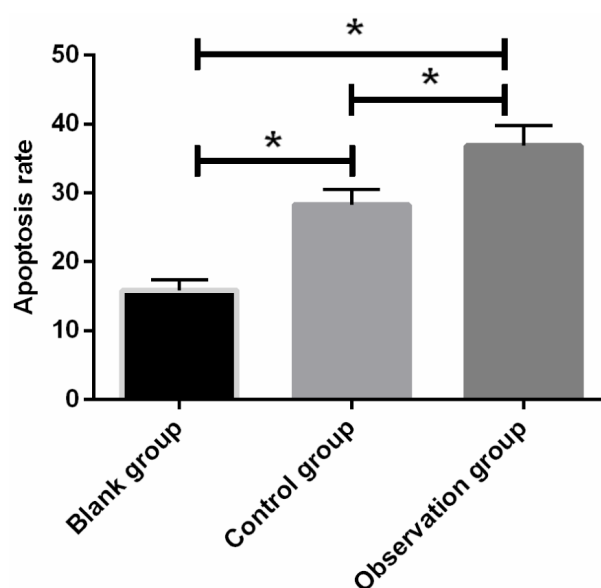


Figure 3. Cell apoptosis. Detection of cell apoptosis by flow cytometry found that the apoptotic ability in the blank group was significantly lower than that in the other two groups ($*p < 0.05$), and that in the control group was significantly lower than that in the observation group ($*p < 0.05$).

Table 4. Cell invasion

Group	Cell membrane number	F	p value
Blank group	33.65 ± 5.69		
Control group	15.86 ± 4.35 ^a	32.612	0.001
Observation group	6.39 ± 1.25 ^{ab}		

"a" shows that there is a difference compared with the blank group ($p < 0.05$), and "b" indicates that there is a difference compared with the control group ($p < 0.05$).

Table 5. Cell apoptosis (mean ± SD)

Group	Apoptosis rate	F	p value
Blank group	15.84 ± 1.54		
Control group	28.33 ± 2.21 ^a	62.229	0.000
Observation group	36.84 ± 2.98 ^{ab}		

"a" shows that there is a difference compared with the blank group ($p < 0.05$), and "b" indicates that there is a difference compared with the control group ($p < 0.05$).

Table 6. Relative expressions of Bax and Bcl-2 mRNA in cells

Gene	Blank group	Control group	Observation group	F	p value
Bax	1.012±0.035	1.358±0.133 ^a	1.619±0.168 ^{a,b}	17.702	0.003
Bcl-2	1.032±0.027	0.765±0.147 ^a	0.501±0.127 ^{a,b}	16.493	0.004

"a" shows that there is a difference compared with the blank group ($p < 0.05$), and "b" indicates that there is a difference compared with the control group ($p < 0.05$).

was significantly lower than that in the observation group with a statistical difference ($p < 0.05$) (Figure 3, Table 5).

Relative expressions of Bax and Bcl-2 mRNA in cells

Detection the relative expressions of Bax and Bcl-2 mRNA in the cells of each group found that there was significant difference in the expressions of Bax and Bcl-2 mRNA between the groups ($p < 0.05$). The expression of Bcl-2 in the cells of the observation group was significantly higher than that of the blank group and the control group, and that in the control group was higher than that in the blank group with a statistical difference ($p < 0.05$). The expression of Bax in the cells of the observation group was significantly lower than that of the blank group and the control group, and that in the control group was lower than that in the blank group with a statistical difference ($p < 0.05$) (Table 6).

Discussion

In recent years, CRC has become quite common in the clinic, which may be caused by the changes in people's diet and eating habits and the aging of the population [12]. In European and American countries with developed medical standards, the incidence of CRC in malignant tumors of men ranks third only after lung cancer and prostate cancer, and is second only to breast cancer in females [13], which is also one of the challenges faced by clinicians today.

The mitogen-activated protein kinase (MAPK) pathway is basically involved in all cellular biological function regulation [14-16]. And ERK, as an important member of the MAPK pathway, is phosphorylated by the activation of a variety of growth factors and cytokines and tumor promoters, and bind to transcription factors such as Elk1 and AP1 in the nucleus, thereby promoting transcription and differential expression of the proto-oncogene cyclinD1, which is closely related with cell proliferation [17,18]. Some authors [19] have shown that the expression and phosphorylation expression level of ERK1/2 in breast cancer cells is

higher than that in normal breast epithelial cells, suggesting that abnormal activation of ERK1/2 may play an important role in the occurrence and development of breast cancer. Moreover, studies have shown that the expression of ERK in renal cell carcinoma tissues detected by Western blot is significantly increased, indicating that ERK activation is involved in the occurrence of various tumors [20,21]. PD98059, as a specific inhibitor in the ERK1/2 pathway, has cell permeability and selectivity [22,23]. Therefore, the effects of PD98059 combined with paclitaxel on CRC tissues were assessed in this study to provide a new direction for clinicians.

Results of intervention of PD98059 combined with paclitaxel in human CRC tissue and detection by Western blot and biological function experiments in this study showed that the proliferation of cells in the observation group treated with PD98059 combined with paclitaxel was significantly inhibited and significantly decreased compared with the control group, while the proliferation of cells in the control group treated with PD98059 alone was significantly lower than that in the blank group, which suggests that PD98059 can inhibit the proliferation of CRC cells, and further inhibit the proliferation by combining with paclitaxel. The use of anticancer drugs is to inhibit the proliferation of cancer cells and promote their apoptosis. Therefore, detection of apoptosis by flow cytometry was performed in this study, and the results showed that apoptosis in the observation group and the control group was significantly higher than that in the blank group, and apoptosis in the observation group was higher than that in the control group, which indicates that the combined use of drugs can promote apoptosis of CRC cells. Moreover, expressions of Bax mRNA (Bax is a pro-apoptotic protein) and Bcl-2 mRNA (Bcl-2 is an anti-apoptotic protein) [23] were detected by PCR, and the results showed that the expression of Bax mRNA in the observation group pretreated with PD98059 and paclitaxel was significantly higher than that in the other two groups, while the expression of Bcl-2 mRNA in the observation group was significantly lower than that in the other two groups, indicating that PD98059 inhibitors may interfere with the

expression of Bax and Bcl-2 mRNA in the cells by some means, thus, promoting apoptosis of cancer cells. In the study of Xu et al [24], it was shown that the use of PD98059 promoted apoptosis of colon cancer cells treated with paclitaxel, which was a good example and evidence and validation of our results. The results of cell invasion test showed that the cell perforation in the observation group and the control group was significantly lower than that in the blank group, and that in the observation group was lower than in the control group. And last, the ERK1/2 and p-ERK1/2 protein in cells was detected and found that there was no difference in the expression of ERK1/2 protein in each group, while the expression of p-ERK1/2 protein in the observation group was significantly lower than that in the control group and the blank group. Friedman et al [25] showed that PD98059 could only inhibit the activation of downstream ERK, but could not regulate the expression of ERK, suggesting that PD98059 indirectly inhibited the ERK1/2 pathway by inhibiting the phosphorylation of ERK1/2, while the inhibition of ERK1/2 phosphorylation by combination of paclitaxel was more obvious, indicating that paclitaxel may also inhibit the phosphorylation of ERK1/2, which has been confirmed in the study of McKeigan et al [26]. Therefore, it is speculated that ERK1/2 signaling pathway-related proteins were inhibited by PD98059 in order to participate in the proliferation, apoptosis and invasion of CRC cells.

However, there are still some defects in the research. Firstly, the effect of different concentrations of drugs on CRC cells has not been established to

find out the best effect. Secondly, this study was conducted *in vitro* and was not piloted in the clinic. Therefore, we hope to establish groups with different concentrations to find out the best therapeutic concentration in future researches, and conduct clinical trials.

In conclusion, PD98059 inhibitor combined with paclitaxel can affect the expression of ERK1/2 in ERK1/2 signaling pathway, effectively inhibit the proliferation and invasive abilities of CRC cells, increase the apoptotic ability of CRC cells, and is expected to become a potential drug for clinical treatment of CRC.

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Authors' contributions

YL helped with transwell assay. YL and QY performed flow cytometry and western blot. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Shenzhen People's Hospital Longhua Branch.

Conflict of interests

The authors declare no conflict of interests.

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