Research Paper

Remifentanil Restrains Malignant Behaviors of Colorectal Cancer Cells through Modulation of the Chemokine Ligand 2/C-C Chemokine Receptor 2 Signaling

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Remifentanil has anti-tumor effects, but its effects on colorectal cancer are unclear. Therefore, the present study investigated whether remifentanil mediates colorectal cancer cell malignant behaviors through the modulation of chemokine ligand 2-C-C chemokine receptor 2 signaling. SW480 cells were divided into control, low remifentanil dose (0.5 ng/ml), medium remifentanil dose (5.0 ng/ml), high remifentanil dose (50.0 ng/ml), and high remifentanil dose+GW0742 (1.0 µM) groups. Detection of SW480 cell proliferation, apoptosis, and migration was performed by cell counting kit-8, flow cytometry, and wound-healing assays. Bcl-2-associated X protein, proliferating cell nuclear antigen, programmed death-ligand 1, B-cell lymphoma 2, chemokine ligand 2, and C-C chemokine receptor 2 protein levels were examined by Western blot. Remifentanil inhibited SW480 cell proliferation and migration, and facilitated SW480 cell apoptosis in a dose-dependent pattern, accompanied by a striking decrease in proliferating cell nuclear antigen, programmed death-ligand 1, and B-cell lymphoma 2 protein levels and an overt elevation in Bcl-2-associated X protein levels. Bindarit had similar effects as remifentanil, and GW0742 attenuated remifentanil-mediated effects on SW480 cell proliferation, migration, and apoptosis. Remifentanil and bindarit inhibited chemokine ligand 2 and C-C chemokine receptor 2 protein levels, but GW0742 attenuated remifentanil-mediated effects on chemokine ligand 2 and C-C chemokine receptor 2 protein levels. Remifentanil may inhibit colorectal cancer malignant behaviors by inhibiting the chemokine ligand 2/ C-C chemokine receptor 2 signaling.

Key words: Colorectal cancer, remifentanil, C-C chemokine receptor 2, chemokine ligand 2, Western blot

Colorectal Cancer (CRC) is a common malignancy in the digestive system, including both colon and rectum cancers. The number of CRC incidents in 2020 exceeded 1.93 million cases within worldwide, accounting for 9.7 % of diagnosed malignancies[1]. It is predicted that new cases of CRC will reach 2.5 million worldwide by 2035[2]. As the clinical symptoms of CRC are insidious in the early stage, a large number of patients have already developed into intermediate and advanced stages with poor prognosis when they are first diagnosed[3,4]. The prevalence of CRC tends to be younger, with the cause of death closely related to tumor invasion and distant metastasis[4]. Although aberrant alterations in tumor-suppressive genes and oncogenes have been reported to drive CRC carcinogenesis and progression[5,6], the exact molecular mechanisms involved in CRC pathogenesis and metastasis are unclear. Therefore, searching for molecular mechanisms associated with CRC advancement is a hot topic of current research.

Remifentanil (Rem) is a commonly used anesthetic drug with rapid onset of action and perfect functional recovery[7]. In addition, Rem can be used as an analgesic[8]. Findings have shown that Rem can inhibit postoperative immunosuppression in tumor patients, enhance the body’s immune ability and inhibit tumor metastasis[9]. Some studies have shown that Rem has the anti-proliferative and apoptosis-promoting functions in lung cancer cells and hepatocellular carcinoma[10,11]. As a commonly used clinical drug, the action mechanism of Rem

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on CRC is still unclear.

Chemokine C-C Motif Ligand 2 (CCL-2) is involved in the regulation of the tumor[12]. CCL-2 works together with C-C Chemokine Receptor 2 (CCR2) to mediate the chemotaxis of some tumor-associated cells, thus promoting the progression of tumor cells[13]. Multiple reports have demonstrated that CCL2 and CCR2 are highly expressed in diverse malignant tumors, and CCL2 binds to CCR2 and activates tumor cells through the CCL2/CCR2 molecular axis, promoting malignant biological processes[14-16]. Moreover, the CCL2/CCR2 axis also recruits Tumor-Associated Macrophages (TAMs) into tumor tissues and promotes tumor progression[15,17]. Obstruction of the CCL2/CCR2 axis promises to be an innovative tactic for malignant tumors.

Therefore, the effect of Rem on CRC cell proliferation, apoptosis, and migration was explored based on the CCL2/CCR2 signaling axis.

MATERIALS AND METHODS

Cell culture:

Human CRC cell line SW480 (Baililai Biotechnology Co., Ltd., Shanghai, China) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Cat No: FK-QV509, Joe feather Biotechnology Co., Ltd., Shanghai, China) containing 10 % fetal bovine serum at 37° with 5 % Carbon dioxide (CO_2). When the cell growth density reached >70 %, the cells were collected and passaged for subsequent studies.

Reagents and instruments:

The Cell Counting Kit-8 (CCK-8) (Cat. No: BN15201-20x500T) from Bairui Biotechnology Co., Ltd., (Beijing, China); Rem (Cat. No: CNPq H20030199) from Yichang Humanwell (China); the annexinV-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis detection kit (Cat. No: FP2050M) from Gaochuang Chemical Technology Co., Ltd., (Shanghai, China); Bindarit (Cat. No. ab143292) from BIOCREATIVE (Beijing, China); total protein extraction kit (Cat. No. MBP0314-100T) from Bihe Biochemical Technology Co., Ltd (Shanghai, China); GW0742 (Cat. No. M2742-50 mg) from Kehao Biotechnology Co., Ltd (Wuhan, China); the Bicinchoninic Acid (BCA) kit (Cat. No. YT8173) from Ita Biotechnology Co., Ltd (Beijing, China); an antibody against BCL-2-Associated X Protein (BAX) (Cat. No. FS-1098) from Fusheng Industrial Co., Ltd (Shanghai, China); antibodies against Programmed Death-Ligand 1 (PD-L1) (Cat. No. PL0403171), B-Cell Lymphoma 2 (Bcl-2) (Cat. No: 251711), CCL2 (Cat. No: PL0304039), and CCR2 (Cat. No: 250580) from Otwo Biotech (Shenzhen, China); an antibody against Proliferating Cell Nuclear Antigen (PNCA) (Cat. No: orb824257) from Biorbyt (Wuhan, China); skimmed milk powder (Cat. No: BB-35122) from Bestbio (Shanghai, Cina); crystal purple (Cat. No: Js19004-25g) Yanjin Biotechnology Co., Ltd., (Shanghai, China); gel imager (Cat. No: EBOX CX5) from Hecheng Technology Co., Ltd., (Wuhan, China); the CO2 cell culture incubator (Thermo) from Fuze Trading Co., Ltd., (Shanghai, China); the flow cytometer (BD FACSCALIBUR) from Exsson (Beijing, China); the microplate reader (Cat. No: 51119080) from Shanghai Sunshine Biotechnology Co., Ltd., (Shanghai, China).

Cell grouping:

Normal cultured SW480 cells were used as the control group. SW480 cells cultured in a medium containing different concentrations of Rem were divided into the low-dose Rem group (0.5 ng/ml, L-Rem), the medium-dose Rem group (5.0 ng/ml, M-Rem), and the high-dose Rem group (50.0 ng/ml, H-Rem), respectively. SW480 cells cultured with Bindarit (the CCL2/CCR2 signaling inhibitor) at a concentration of 300 μM were divided into the Bindarit group. SW480 cells cultured with Rem at a concentration of 50.0 ng/ml and GW0742 (the CCL2/CCR2 signaling activator) at a concentration of 1.0 μM were divided into the H-Rem+GW0742 group.

Detection of SW480 cell proliferation:

Trypsin digestion was executed when SW480 cells in each group grew to the logarithmic phase. Digested cells were cultured for 48 h. Following incubation with the CCK-8 solution, the absorbance values at 450 nm were detected on a microplate reader.

Measurement of SW480 cell apoptosis:

SW480 cells cultured for 48 h were digested with trypsin, followed by washing with pre-cooled Phosphate Buffer Solution (PBS). The precipitate obtained by centrifugation was supplemented

with binding buffer, followed by the addition of AnnexinV-FITC and PI. The reaction was carried out under dark conditions for 10 min, and apoptosis was detected by flow cytometry.

Determination of the migratory ability of SW480 cells:

SW480 cells grown to log phase were inoculated in 6-well plates. 24 h later, the 6-well plates were lightly scratched with the tip of the pipette tip to create a wound. The cell migration was observed under an inverted microscope at 0 h and 48 h after scratching, respectively. The healing rate (%) of the scratches was also calculated.

Western blot:

The cultured SW480 cells from each group were collected, followed by the addition of a certain amount of protein lysate on ice for 30 min. The lysed cells were centrifuged, the supernatant was collected, and the total protein of the cells in each group was extracted with the total protein extraction kit, and their concentrations were determined according to the BCA kit instructions. The protein extracts were separated using Sodium dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel electrophoresis, followed by transfer to Polyvinylidene Difluoride (PVDF) membrane. After sealing using 5 % skimmed milk powder, the membranes were incubated with primary antibodies against BAX, PNCA, PD-L1, Bcl-2, CCL2, CCR2, or Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH). After overnight incubation at 4°, the membranes were incubated with secondary antibodies. The membrane was visualized using a chemiluminescent detection system.

Western blot:

All experiments were performed in triplicate. Statistical analysis were performed using GraphPad Prism software (version 7.0, United States of America (USA)). All data are expressed as mean±standard deviation. The two groups were compared using the t-test or Mann-Whitney U-test, depending on normal distribution. Other experiments used ordinary one-way Analysis of Variance (ANOVA). $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

A gradual decrease in the Optical Density (OD) values of SW480 cells was observed in the L-Rem, M-Rem, and H-Rem groups in comparison to the control group with an increase in the Rem dose ($p<0.05$). The inhibitor of CCL2/CCR2 signaling, bindarit, resulted in a conspicuous reduction in the OD values of SW480 cells relative to the control group ($p<0.05$). Notably, GW0742, an agonist of CCL2/CCR2 signaling, elevated the OD values of H-Rem treated SW480 cells significantly compared to the H-Rem group ($p<0.05$) (Table 1).

In contrast to the control group, L-Rem, M-Rem, and H-Rem resulted in a gradual increase in SW480 cell apoptosis with growing doses of Rem ($p<0.05$). Bindarit also led to an improvement in SW480 cell apoptosis in comparison to the control group ($p<0.05$). It was noteworthy that the usage of GW0742 significantly declined H-Rem treated SW480 cell apoptosis *vs.* the H-Rem group ($p<0.05$) (fig. 1 and Table 2).

The migratory ability of SW480 cells was gradually inhibited by Rem with the increase of dose ($p<0.05$). Bindarit also inhibited SW480 cell migration *vs.* the control group ($p<0.05$). Comparing with the H-Rem group, GW0742 promoted the migration of H-Rem treated SW480 cells ($p<0.05$) (fig. 2 and Table 3).

TABLE 1: PROLIFERATIVE CAPACITY OF SW480 CELLS WAS INHIBITED BY REM THROUGH INHIBITION OF THE CCL2/CCR2 SIGNALING

Group	OD values
Control	0.97±0.18
L-Rem	$0.72±0.16^{*}$
M-Rem	$0.43±0.13^{*\#}$
H-Rem	$0.12±0.04^{*\#\&}$
Bindarit	$0.13±0.03^{*\#\&}$
H-Rem+GW0742	$0.81±0.17^{@}$

Note: $^{*}p<0.05$ *vs.* control; $^{\#}p<0.05$ *vs.* L-Rem; $^{\&}p<0.05$ *vs.* M-Rem and $^{@}p<0.05$ *vs.* H-Rem

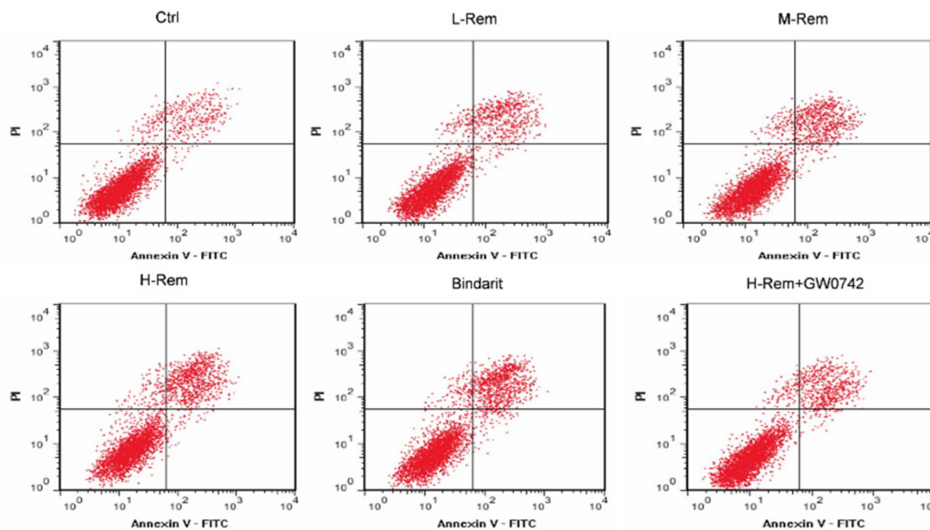

Fig. 1: Detection of apoptosis by flow cytometry

TABLE 2: REM FACILITATED SW480 CELL APOPTOSIS

Group	Apoptosis (%)
Control	12.37±1.76
L-Rem	19.52±2.34*
M-Rem	26.51±2.81*#
H-Rem	32.67±3.42*#&
Bindarit	33.05±3.67*#&
H-Rem+GW0742	18.26±1.98@

Note: *p<0.05 *vs*. control; #p<0.05 *vs*. L-Rem; &p<0.05 *vs*. M-Rem and @p<0.05 *vs*. H-Rem

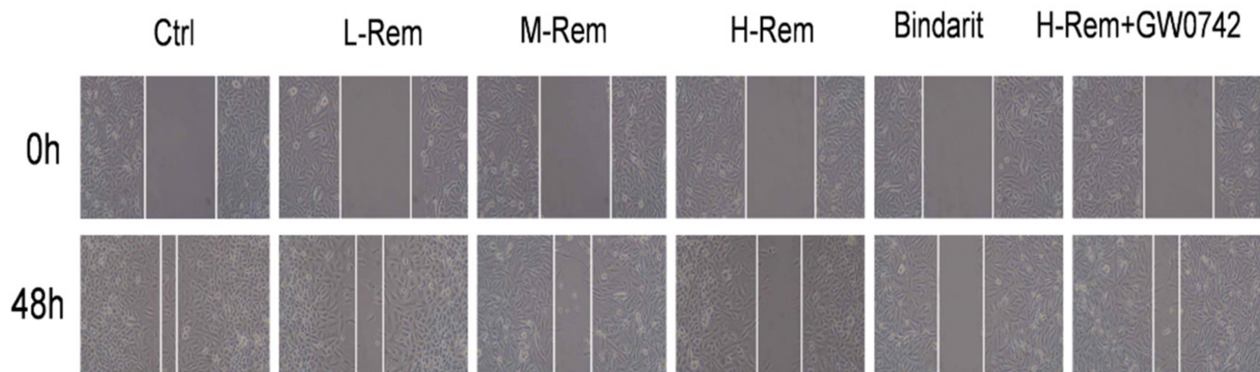

Fig. 2: Detection of SW480 cell migration by wound healing assays

TABLE 3: REM REPRESSED SW480 CELL MIGRATION

Group	Wound healing rate (%)
Control	76.28±8.35
L-Rem	63.17±7.16*
M-Rem	50.16±6.98*#
H-Rem	36.59±4.71*#&
Bindarit	37.61±4.74*#&
H-Rem+GW0742	63.69±7.26@

Note: *p<0.05 vs. control; #p<0.05 *vs*. L-Rem; &p<0.05 *vs*. M-Rem and @p<0.05 *vs*. H-Rem

By comparison with the control group, Rem caused a prominent improvement in BAX protein levels as well as a reduction in PNCA, PD-L1, and Bcl-2 protein levels in SW480 cells in a concentration-dependent model ($p<0.05$). The effects of bindarit on BAX, PNCA, PD-L1, and Bcl-2 proteins were consistent with Rem ($p<0.05$). However, GW0742 impaired the effects of H-Rem on the above protein alterations ($p<0.05$) (fig. 3 and Table 4).

In contrast to the control group, Rem reduced CCL2 and CCR2 protein levels in a concentration-dependent model ($p<0.05$). Consistently, bindarit also reduced CCL2 and CCR2 protein levels ($p<0.05$). However, GW0742 attenuated the effect of H-Rem on the above-mentioned protein changes ($p<0.05$) (fig. 4 and Table 5).

CRC is the 4th and 5th causes of malignancy-related deaths in women and males in our country[18]. There are many factors contributing to CRC, which are generally believed to be related to heredity and lifestyle habits. Surgical resection is the main treatment modality used in clinical practice, with targeted therapy as well as chemotherapy as adjuvant treatments[19]. Owing to the improvement of surgical techniques and advances in adjuvant treatments such as chemotherapy, the 5 y survival of CRC patients has also increased from 50 % to 65 %, but it still fails to achieve the expected results[20]. The molecular mechanisms involved in CRC development are still unclear; therefore, in-depth studies on the underlying mechanisms associated with its development are needed.

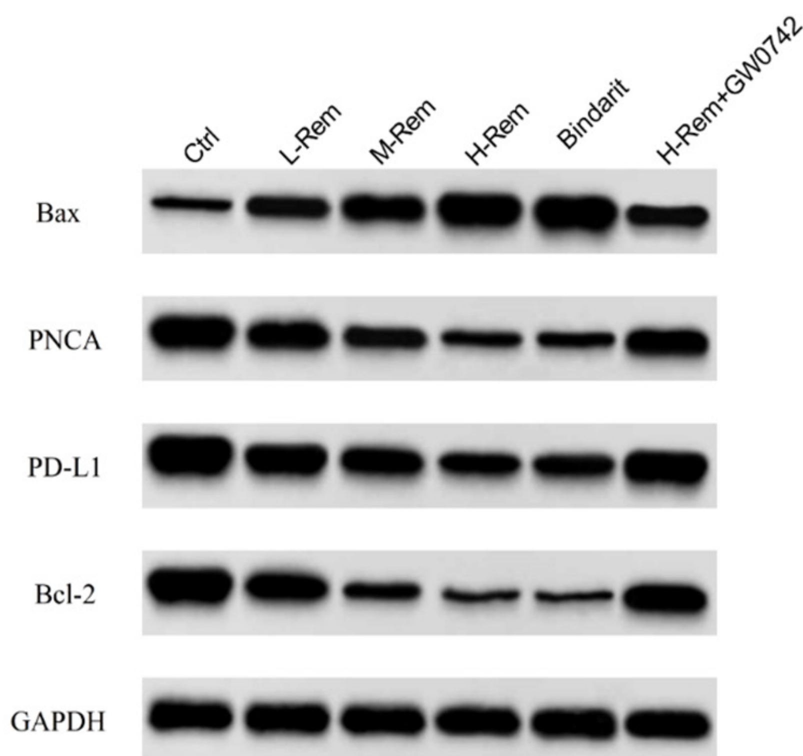

Fig. 3: Western blot detection of BAX, PNCA, PD-L1, and Bcl-2 protein levels in SW480 cells

TABLE 4: EFFECTS OF REM ON APOPTOSIS-RELATED PROTEINS IN SW480 CELLS

Group	BAX	PNCA	PD-L1	Bcl-2
Control	0.37±0.07	1.46±0.16	1.79±0.18	1.46±0.29
L-Rem	0.75±0.13*	1.16±0.13*	1.36±0.14*	1.05±0.25*
M-Rem	1.06±0.18*#	0.81±0.11*#	1.02±0.11*#	0.56±0.15*#
H-Rem	1.52±0.21*#&	0.46±0.06*#&	0.65±0.07*#&	0.19±0.05*#&
Bindarit	1.59±0.23*#&	0.49±0.08*#&	0.68±0.09*#&	0.18±0.04*#&
H-Rem+GW0742	0.70±0.09@	1.08±0.12@	1.32±0.14@	1.19±0.27@

Note: *$p<0.05$ *vs.* control; #$p<0.05$ *vs.* L-Rem; &$p<0.05$ *vs.* M-Rem and @$p<0.05$ *vs.* H-Rem

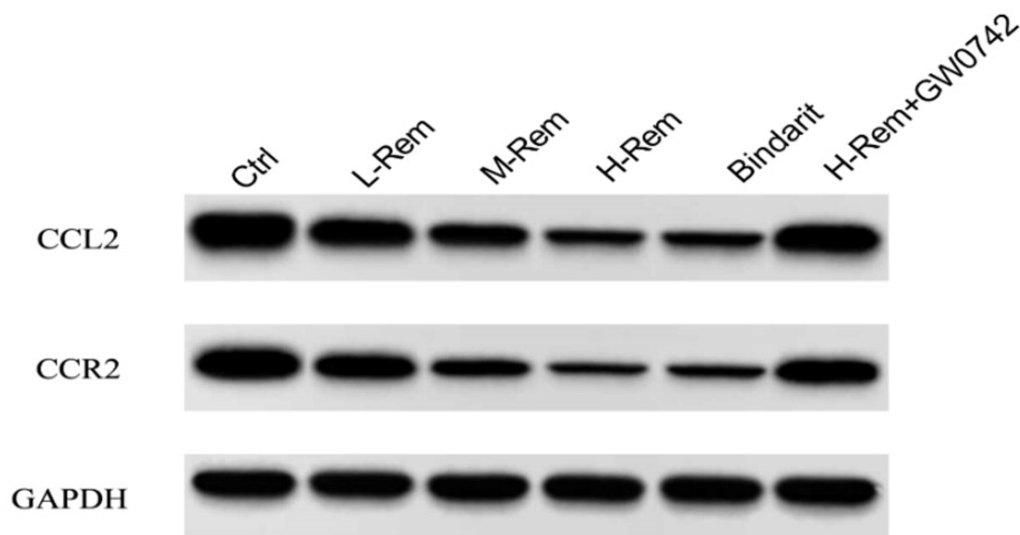

Fig. 4: Western blot detection of CCL2 and CCR2 protein levels in SW480 cells

TABLE 5: EFFECTS OF REM ON THE CCL2/CCR2 SIGNALING IN SW480 CELLS

Group	CCL2	CCR2
Control	1.57±0.29	1.32±0.25
L-Rem	1.15±0.23*	0.91±0.16*
M-Rem	0.76±0.17*#	0.57±0.15*#
H-Rem	0.33±0.10*#&	0.26±0.07*#&
Bindarit	0.37±0.12*#&	0.30±0.09*#&
H-Rem+GW0742	1.25±0.26@	0.95±0.21@

Note: *p<0.05 *vs.* control; #p<0.05 *vs.* L-Rem; &p<0.05 *vs.* M-Rem and @p<0.05 *vs.* H-Rem

Rem, as an opioid, can be hydrolyzed by non-specific esterases[21]. Previously, Rem has been used as an anesthetic. Recently, it has been found to play a role in tumors. A study reported that Rem exhibits an anti-tumor effect in gastric cancer[22]. In HepG2 cells, Rem restrains cell proliferation and accelerates cell apoptosis[23]. Here, Rem decreased OD values, scratch healing rates, and the levels of PNCA and Bcl-2 proteins, but increased apoptosis rates and BAX protein levels, indicating that Rem can promote SW480 cell apoptosis and restrain SW480 cell proliferation and migration. PD-L1, the ligand of PD-1, is a member of tumor immunosuppression[24]. PD-1 participates in the immune check, and overexpression of PD-1 in tumor cells increases cell immune tolerance, resulting in a favorable immune escape for tumor cells[25]. In this study, Rem was found to reduce PD-L1 protein levels in a dose-dependent pattern, suggesting that Rem can downregulate PD-L1 expression and restrain SW480 cells from immune escape.

PD-1 can play a role in the CCL2/CCR2 signaling axis, thus contributing to the immune escape of cancer cells[13]. CCL2 is an important factor associated with the immune system, and the CCL2/CCR2 signaling is thought to be closely related to tumor progression, and this signal is frequently used in clinical practice, such as for early detection and postoperative prediction[26]. Liu *et al.*[2] discovered that the CCL2/CCR2 axis facilitates breast cancer development. Ding *et al.*[27] found that the CCL2/CCR2 axis takes part in lung cancer development. A previous study exposed that the upregulation of CCL2 has a promoting effect on CCR2 expression, resulting in the activation of the CCL2/CCR2 signaling, facilitating melanoma cell proliferation and migration[28]. Our results showed that Rem caused a significant decrease in CCL2 and CCR2 protein levels in SW480 cells, suggesting that Rem may play a role by modulating the CCL2/CCR2 axis. Bindarit (CCL2/CCR2 signaling inhibitor) had a similar effect with H-Rem, whereas H-Rem combined with GW0742 significantly increased CCL2 and CCR2 protein levels compared to H-Rem alone. The addition of GW0742 abolished

Rem-mediated inhibitory effects on SW480 cell proliferation and migration as well as promoting effects on SW480 cell apoptosis.

In summary, Rem might suppress CRC cell proliferation and migration, as well as promote CRC cell apoptosis by repressing the CCL2/CCR2 signaling. However, this study was only conducted on CRC cells *in vitro*, and *in vivo* experiments will be carried out in the future to verify the above results.

Conflict of interests:

The authors declared no conflict of interests.

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