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Original Article

Inhibiting the Proliferation of Colorectal Cancer Cells by Reducing TSPO/VDAC Expression

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Abstract

Background: We aimed to explore the mechanism of the effect of remimazolam (Rem) on the proliferation of colorectal cancer (CRC) cells with CRC as a disease context.

Methods: Translocation protein (TSPO) expression in CRC was determined by Western blotting and qRT-PCR in the Second Affiliated Hospital of Qiqihar Medical University from March 2019 to February 2022. TSPO-interacting proteins were predicted through string database. The proliferation was measured by CCK-8 and 5-ethynyl-2-deoxyuridine (EDU). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) and clonal colony on cells were formed to screen for the optimal concentration of Rem and to detect the viability. The expression of apoptosis-related proteins, Bcl-2 and P53, was determined by qRT-PCR and Western blotting. The effect of Rem on the expression of tumor markers, CEA and CA19-9, in CRC was examined through ELISA.

Results: TSPO expression in CRC tissues and cells was higher than that in ANT samples and normal intestinal epithelial cells. Over-expression of TSPO promoted the proliferation of HCT116 and the expression of tumor markers CEA and CA19-9 and inhibited the apoptosis of HCT116. Interference with TSPO inhibited the proliferation of HCT116 and the expression of CEA and CA19-9 and promoted the apoptosis of HCT116. 1 μg/mL Rem could inhibit the viability of HCT116, the proliferation of HCT116 and the expression of CEA and CA19-9, and improve the apoptosis of HCT116. TSPO could interact with VDAC and affect its protein expression, and Rem could inhibit the proliferation and the expression of CEA and CA19-9 through the TSPO/VDAC pathway, to promote its apoptosis.

Conclusion: Rem affects the proliferation of CRC cells by inhibiting the TSPO/VDAC pathway.

Keywords: Colorectal cancer; Remimazolam; Translocation protein

Introduction

Colorectal cancer (CRC) is the third most common cause of death worldwide. Among the people diagnosed with metastatic CRC, the number of those who survive longer than 5 years was less than 20% (1). The standard treatments for CRC are mainly surgery, chemotherapy and radiotherapy; however, they will have many side effects, such as leukopenia and neutropenia (2). Therefore, it is urgent to find new and effective therapies for treating CRC.

The role of Benzodiazepines (BZDs) in CRC is gradually discovered (3). The over-expression of benzodiazepine receptor (PBR) is a new independent prognostic marker for CRC in stage III (4). One of the peripheral BZD receptors, 18 kDa translocation protein (TSPO), localized in the outer membrane of the mitochondria, is up-regulated in CRC (5,6), and TSPO is involved in the apoptosis-regulated of HT29 in CRC cells (7). The pharmacological effects of BZDs require both TSPO and voltage-dependent anion channel (VDAC) -bound complexes to play a role (8). Moreover, TSPO and VDAC and adenine nucleotide translocase (ANT) to maintain the balanced of mitochondrial respiratory chain (9) and regulate functions of mitochondrion (10). Therefore, BZDs can regulate the functions of mitochondrion through the TSPO/VDAC pathway, to exert their pharmacological effects in the disease control. Both BZDs and TSPO function in CRC, and BZDs are required to target TSPO, therefore, targeting the TSPO/VDAC pathway can help in controlling the development of CRC.

Remimazolam (Rem) is a newer BZD (11). Rem has an onset time of 1-3 min (12) and a terminal half-life of 0.75 h (13) and does not accumulate in tissues (14), showing good safety and good drug tolerance in clinical experiments (15). The role of Rem as a new BZD in CRC has not been reported, therefore, Rem will likely be used as a new generation of targeted drugs for the treatment of CRC.

Effective CRC-targeted drugs currently play an important role in the detection and treatment for CRC patients, and searching for new biomarkers can identify the susceptibility or early stages of the disease. Therefore, this study deeply explored the role of Rem in the treatment of CRC, aiming to develop a new molecular pathway for the targeted prediction of CRC, to help in the prediction and prevention of CRC.

Materials and Methods

Collection of clinical samples

Twenty paired CRC tissues and adjunct normal tissues (ANT) samples (with the distance away from the tumor > 5 cm) were collected from patients diagnosed with primary CRC through the evaluation by histopathology at the Second Affiliated Hospital of Qiqihar Medical University between March 2019 and February 2022. All patients had no diseases of heart, brain, kidney or other vital organs, without signs of distant metastasis. No patient received any form of medication and radiation or/and chemotherapy prior to surgery. All patients had signed an informed consent in this study, which has been approved by the Ethics Committee (NO.20190221).

Cell culture

Normal intestinal epithelial cell line, NCM460, and CRC cell line, HCT116, were obtained from the Cell Bank of Wuhan University. Cells were cultured in DMEM high-glucose medium containing 10% FBS (Art. No.: 164210-500). Sterile penicillin and streptomycin (100 μg/mL) were added in the medium. All cells were cultured in an incubator with 5% CO_2.

Cell transfection

TSPO plasmids were used as the over-expression vector, and the over-expression vector (GFP-TSPO) and the control (Vector) were constructed by Triangle Biotechnology Co., Ltd. (Shanghai, China). Sequence of siRNA targets designed for knockdown of TSPO expression (si-TSPO): ACCATTGGGCCTGCTGGTA. The reagent for transfection was Lipofectamine 2000 (Invitrogen Gibco, USA), and the plasmid incubation solution was opti-MEM (Invitrogen Gibco, USA). The mixture was slowly dropped to the surface of cells. After transfection for 4.5h, the medium was replaced with proliferation medium.

Drug treatments

Cells were seeded in six-well plates at 5×10^5 cell/well, which were divided into four groups:

control (0 μg/mL Rem) and three Rem-treated groups (0.1, 1 and 10 μg/mL Rem) (16-19). The optimal inhibitory concentration of Rem on the viability of CRC cells was detected by MTT and cell colonies.

Western blotting

The protein extraction kit (Beyotime, P0033, China) was used for extracting proteins from tissues and cells. After denaturing, SDS-PAGE gel electrophoresis was performed, then the proteins were transferred to PVDF membrane (Millipore, MA, USA, IPFL00010) at the current of 200 mA. Then it was closed with skim milk powder, which was incubated overnight with primary antibodies (TSPO: BS-3674R, Bioss; β-actin: BS-0061R, Bioss; VDAC: S-7647R, Bioss), and then incubated with goat anti-rabbit IGG-HRP (1:5000, Millipore, MA, USA, AP112P) for another 2h. Protein expression was finally detected by ECL-Western Blotting Substrate Kit (Abnova, KA3725) and chemiluminometry image system (Fusion Solo, Villber Lourmat).

qRT-PCR

Total RNA was extracted with the RNA extraction kit (Thermo, USA). Templates were synthesized according to the operation requirements of reverse transcription kit (Thermo, USA). When the concentration of the reverse transcribed cDNA strand was determined by the Nano instrument, qRT-PCR amplification was performed in the instrument with the TB Green Premix EX Taq II kit (Thermo, USA). The reaction system of qPCR is as follows: 10μl of cDNA, 2 μl for each of gene upstream and downstream primers, 66 μl of ddH2O, and 20 μl of SYBR Green1 dye. The setting procedure is: 95℃ for 5 min, 94℃ for 15s, 55℃ for 30s, 72℃ for 30s, 4℃ for 30min, for 40 cycles. Internal reference primers were synthesized by a company.

The sequences of primers are as follows:

β-actin: F-5CTCCATCCTGGCCTCGCTGT-3,
R-5GCTGTCACCTTCACCGTTCC-3.

TSPO: F-5GCTAGCTTGCAGAAACCCTC-3,
R-5GCTGACCAGTGTAGAGACCC-3.

Bcl-2: F-5GAGGATTGTGGCCTTCTTTG-3,
R-5GTTCCACAAAGGCATCCCAG-3.

P53: F-5GTTTCCGTCTGGGCTTCT-3,
R-5ACCTCAGGCGGCTCATAG-3.

VDAC: F-5AAGGTCTGCAACTATGGGCT-3,
R-5AACACAGCCCAGCCATAGAT-3.

All the primers above were synthesized by a company.

CCK-8

Transfected cells were seeded into 96-well plates, and 15 μl of CCK-8 reagent (10 μL, abcam) was added to each well and incubated for 3h before the absorbance (450nm) was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) and averaged in triplicate.

EDU

Cells were seeded in 96-well plates at 4×10^3 cells/well and transfected. After transfection for 24 h, 10 μM of EDU (C0075S, Beyotime) was added to each well, and incubated at 37℃ for 2 h. The cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After washing out the excess fixation fluid, the membrane was permeabilized with 1 mL of PBS containing 0.3% Triton X-100 at room temperature for 15 min. Finally, the cells were stained with Hoechst 33342 for 10 min, observed and photographed under a microscope (BX63, Olympus). Five groups of cells were randomly selected and counted to count the rate of EDU positive cells.

Detection for formation of cell colonies

One hundred cells were seeded in six-well culture plates, which were cultured after adding the proliferation culture medium, and the formation of colonies was regularly observed during the culture of cells. Colonies with more than 50 cell clones under microscope were fixed, then the cells were stained with 0.1% crystal violet. The number of colonies formed was counted and all experiments were performed in triplicate.

MTT

1 x 10^4 cells were seeded into 96-well plates and cultured for 24h to allow cell adherence, and then

in the absence of serum, the cells were incubated with Rem (0.1, 1 and 10 μg /mL, Rem, CAS:308242-62-8,Shanghai Pfam Pharmaceutical Technology Co., Ltd.) at 37°C for 24h. Then, MTT solution (0.5 mg/ml, abcam) was added and further incubated at 37°C for 3 h. Finally, the absorbance was measured at 580nm.

ELISA

Cells were inoculated into 6-well culture plates. Cells were treated with Rem (1 μg/mL) for 24h, The supernatant was collected and coincubated with HRP-conjugated antibodies. The optical density (OD) at 450 nm was determined after the colouration. Concentrations of carcinoembryonic antigen (CEA) (Fujirebio Diagnostics AB, Sweden) and carbohydrate antigen 199 (CA19-9) (Fujirebio Diagnostics AB, Sweden) in serum was detected.

Construction of protein-protein interaction network

The protein interaction network of TSPO was acquired via String online database (https://string-db.org/). After protein and species selection, the protein-protein interaction network of TSPO was collected, and finally, the network map between the top 10 interacting proteins was obtained.

Data Analysis

Relevant data analysis were performed with Prism GraphPad 7.0 (GraphPad Software, La Jolla, CA, USA). The number of cells were counted and analyzed with Image J (1.4.3.67 Broken Symmetry software), which was also used to count protein gray scale results. Data were expressed as mean ± standard deviation (SD), and t-test was used to analyze differences between groups. Univariate ANOVA test was performed for the difference statistics of data from more than two groups. $^{*}P<0.05$ and $^{**}P<0.01$ indicates a statistical difference in the data.

Results

TSPO expression is specific in colorectal cancer

As shown in Fig. 1A-B, TSPO expression was higher in CRC tissues than that in ANT samples ($P<0.01$). TSPO expression was higher in CRC cell, HCT116, than that in the normal intestinal epithelial cell line, NCM460, (Fig. 1C-D, $P<0.01$). It further confirmed the correlation between TSPO and the development of CRC.

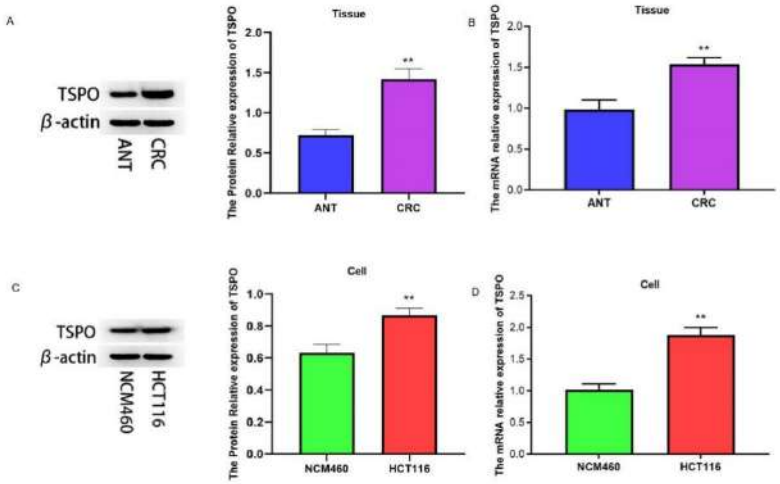

Fig. 1: Correlation between TSPO and CRC

Note: A: Western blotting for the expression difference of TSPO in CRC and ANT; B: qRT-PCR for the expression difference of TSPO in CRC and ANT; C: Western blotting for the expression difference of TSPO in HCT116 and NCM460; D: qRT-PCR for the expression difference of TSPO in HCT116 and NCM460. $^{*}P<0.05$, $^{**}P<0.01$

Effect of TSPO on proliferation, apoptosis and tumor markers

The effect of over-expressed TSPO on the proliferation of HCT116 was determined by CCK-8, the results are shown as Fig. 2A. 1 μg/mL of GFP-TSPO increased the proliferation of HCT116 (P<0.01). In contrast, the transfection of si-TSPO significantly reduced the proliferation of HCT116 (P<0.01). Subsequently, the expression of apoptosis-related proteins, Bcl-2 and P53, was determined by qRT-PCR, and the results are shown as Fig. 2B. Over-expressed TSPO CRC cells had a reduced apoptosis of HCT116 (P<0.01). However, TSPO increased the apoptosis of HCT116 (P<0.01). ELISA was used to detect the changes of expression of CRC tumor markers, CEA and CA19-9. In Fig. 2C, GFP-TSPO promoted the expression of CEA and CA19-9 (P<0.01), while si-TSPO inhibited CEA and CA19-9 (P<0.01). Based on the above results, the changes in TSPO expression can affect the proliferation and apoptosis of CRC cells, and the expression of tumor markers.

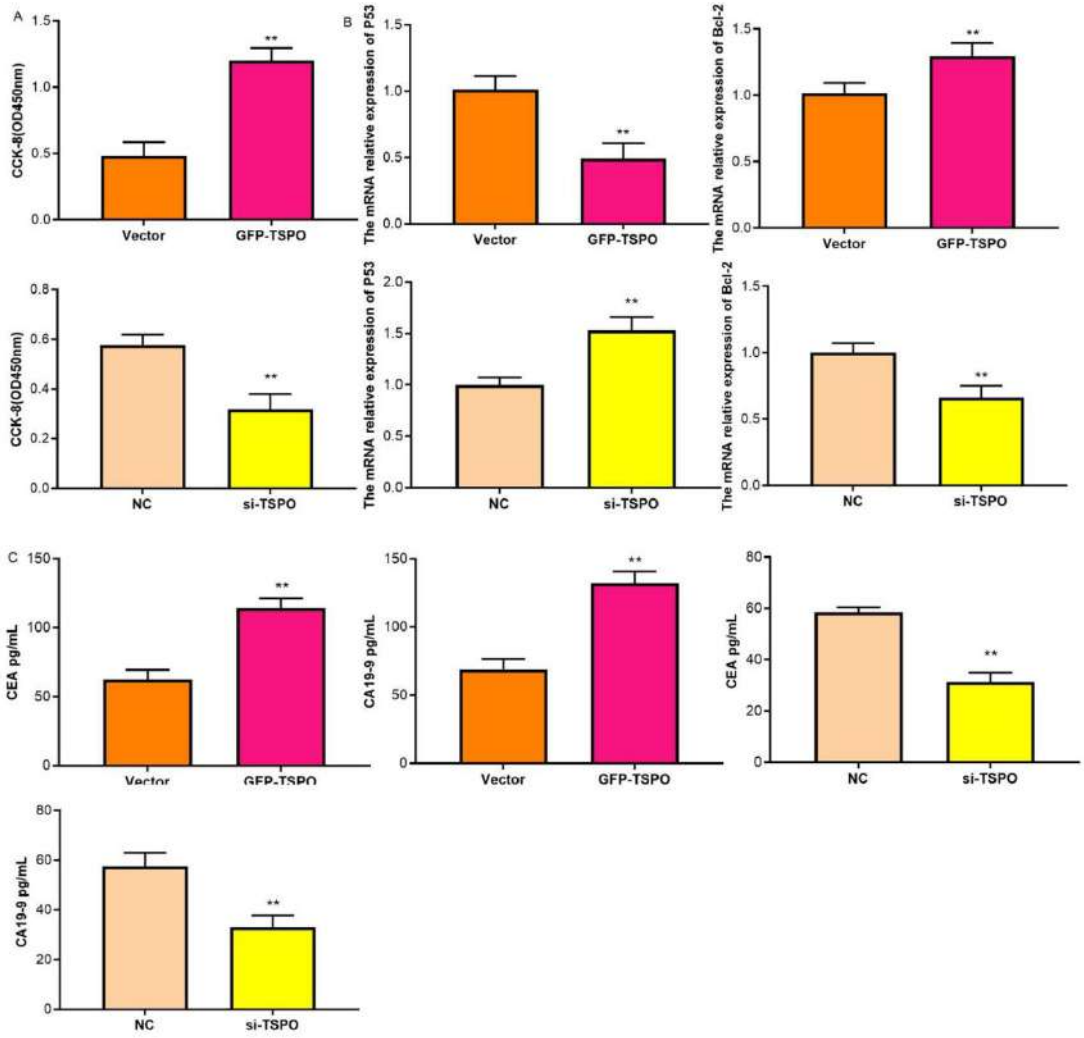

Fig. 2: Effect of TSPO on the proliferation and apoptosis of HCT116, as well as the expression of CEA and CA19-9

Note: A: CCK-8 for TSPO; B: qRT-PCR for P53 and Bcl-2 expression; C: ELISA for CEA and CA19-9 expression. N=3, $^{*}P$<0.05, $^{**}P$<0.01

Effect of Remimazolam on colorectal cancer cells

The optimal drug concentration of Rem acting on HCT116 was first selected. According to MTT analysis, the viability gradually decreased with the increase of Rem action concentration compared with the control cells without Rem-treatment (0 μg/mL). However, its weakening trend is smaller. When Rem treated HCT116 at 1 μg/mL, a slight decrease in the viability was observed (Fig. 3A, $P<0.01$). However, when the concentration of Rem increased to 10 μg/mL, the decreasing trend of viability was slowed down, it could be seen that the concentration of Rem at 1 μg/mL had a great effect on the viability of HCT116. The same results of cell cloning experiments showed that Rem at 1 μg/mL could significantly reduce the formation of clonal cell colonies of HCT116 (Fig. 3B, $P<0.01$), so Rem at 1 μg/mL was chosen as the optimal drug concentration.

HCT116 were treated with Rem at 1 μg/mL for 24h, and the effect of Rem on the proliferation was detected by CCK-8 and EDU. In Fig. 3C-D, Rem at 1 μg/mL significantly inhibited the proliferation of HCT116 ($P<0.01$). Rem at 1 μg/mL significantly promoted the apoptosis of HCT116 (Fig. 3E, $P<0.01$). Similarly, ELISA showed that Rem at 1 μg/mL significantly reduced the expression CEA and CA19-9 compared with controls (Fig. 3F, $P<0.01$). The above results indicated that Rem at 1 μg/mL inhibited the proliferation of HCT116.

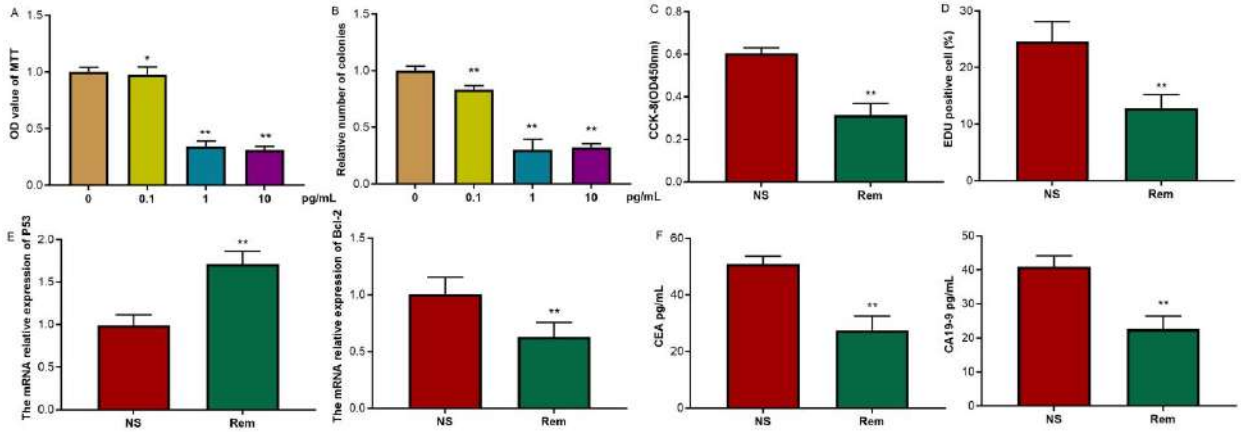

Fig. 3: Effect of Remimazolam medication on colorectal cancer cells

Note: A: MTT for the effect of Rem on the viability of cells; B: Detection on the formation of cell colonies for the effect of Rem on the formation of cell clone; C: CCK-8 for the effect of Rem on the proliferation of HCT116; D: EDU for the effect of Rem on the proliferation of HCT116; E: qRT-PCR for the effect of Rem on P53 and Bcl-2 expression; F: ELISA for the effect of Rem on the expression of CEA and CA19-9. N=3, $^{*}P<0.05$, $^{**}P<0.01$

Targeted regulatory molecules of TSPO in colorectal cancer cells

The String online database (https://cn.string-db.org/) was adopted. The result showed that TSPO can interact with VDAC in CRC cells (Fig. 4A). The regulatory effect of TSPO on VDAC expression was tested by Western blotting, and the result showed that GFP-TSPO promoted VDAC expression, while si-TSPO interfered with VDAC expression in HCT116 (Fig. 4B, $P<0.01$), indicating that TSPO on CRC may work by targeting VDAC.

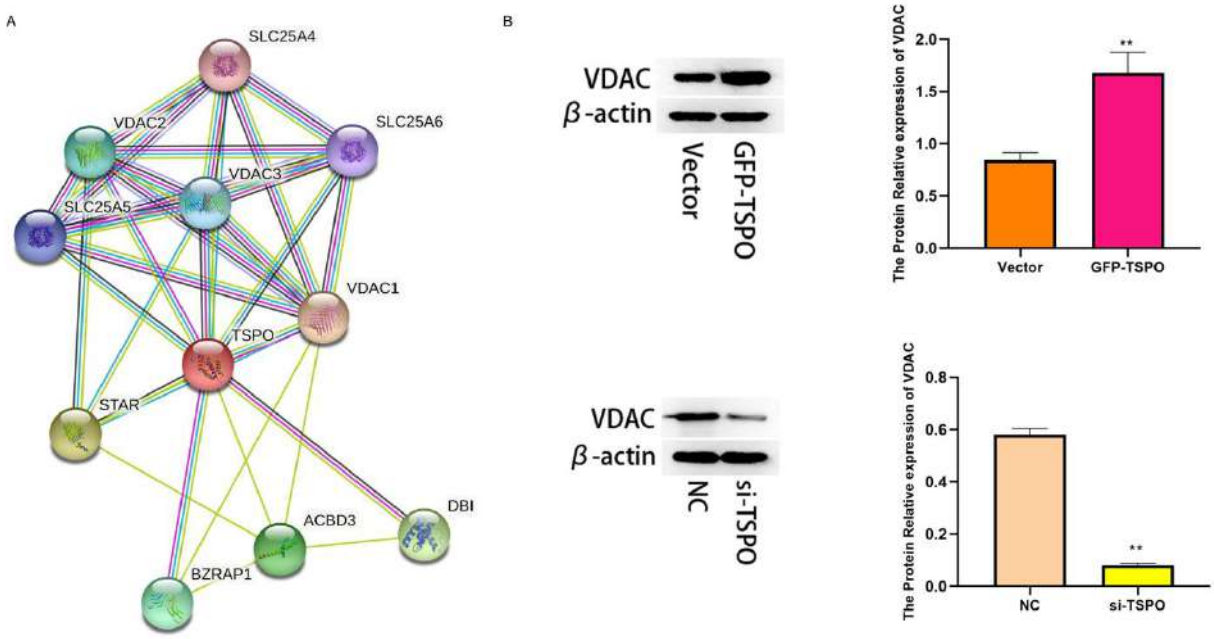

Fig. 4: Targeted regulation of VDAC by TSPO
Note: A: String online database for the prediction of TSPO interacting proteins; B: Western blotting for the regulation of TSPO on VDAC expression. N=3, $^{*}P<0.05$, $^{**}P<0.01$

Remimazolam affects the progression of colorectal cancer by inhibiting TSPO/VDAC

qRT-PCR showed that Rem treatment inhibited the expression of TSPO and VDAC, while after over-expression of TSPO, the inhibitory effect of Rem on TSPO and VDAC was weakened (Fig. 5A, B, $^{*}P<0.05$, $^{**}P<0.01$), suggesting that the pharmacological effects of Rem in HCT116 are related to the TSPO/VDAC pathway. As shown in Fig. 5C. Rem inhibited the proliferation of HCT116, while GFP-TSPO significantly promoted the proliferation of HCT116 ($^{**}P<0.01$), which is consistent with previous results. When HCT116 were simultaneously administered with Rem and over-expressed for TSPO, the proliferation of HCT116 was reduced ($^{**}P<0.01$), suggesting that the promotion effect of TSPO on the proliferation of HCT116 can be inhibited by Rem. Results of apoptosis detection was consistent with this. The inhibition of TSPO on HCT116 apoptosis can be relieved by Rem (Fig. 5D, $^{*}P<0.05$, $^{**}P<0.01$);. Similarly, the results of tumor markers were consistent with this conclusion, the promotion of TSPO on CEA and CA19-9 can be inhibited by Rem (Fig. 5E, $^{*}P<0.05$, $^{**}P<0.01$).. However, when TSPO was simultaneously over-expressed and treated with Rem, the expression of CEA and CA19-9 was found to decrease due to the action of Rem (Fig. 5E,$^{*}P<0.05$, $^{**}P<0.01$). Combined with the above results, Rem inhibited the proliferation of HCT116 and the expression of tumor markers, and promoted the apoptosis, which acts through targeted inhibition of the TSPO/VDAC pathway.

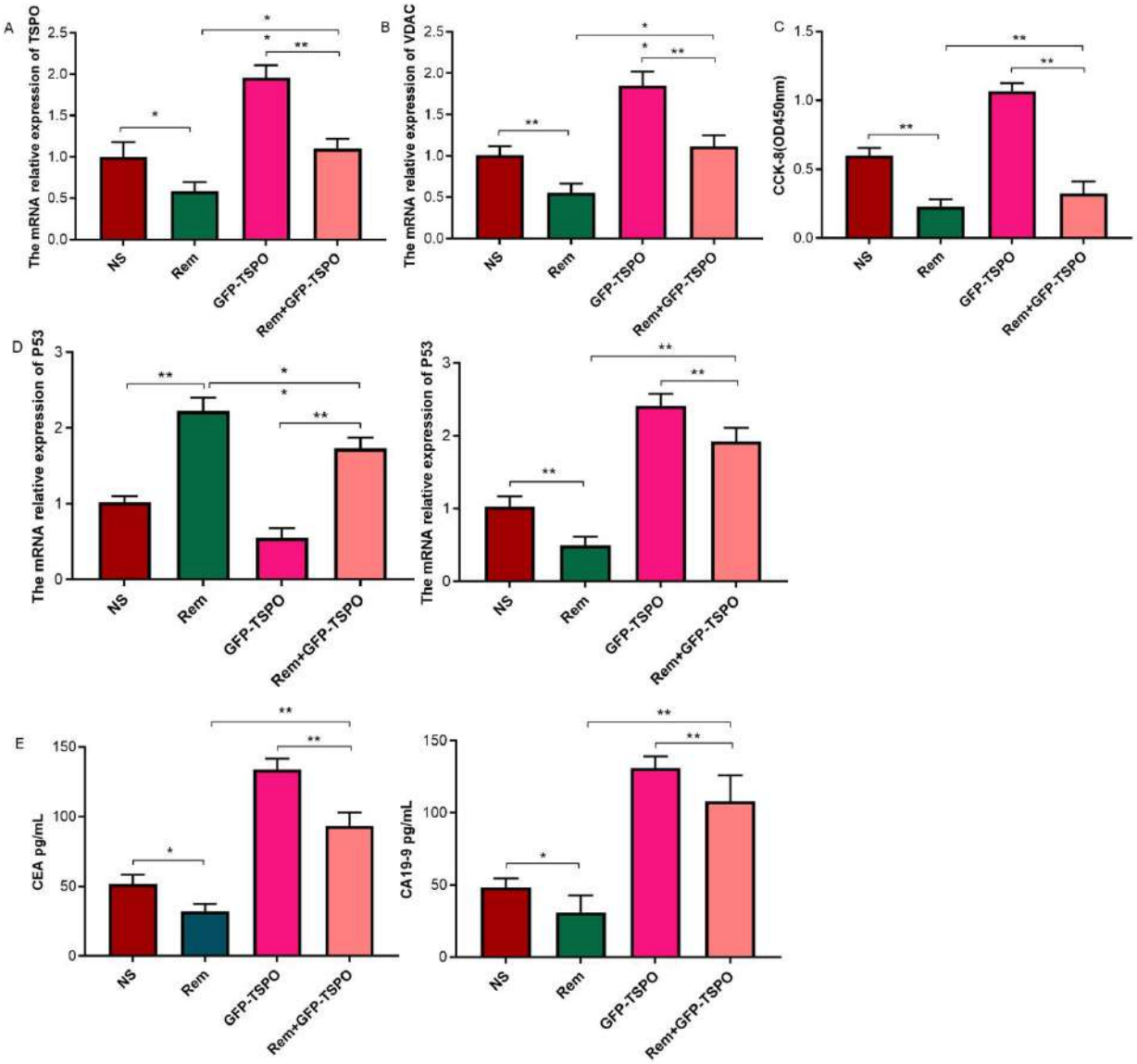

Fig. 5: Remimazolam inhibited the progression of colorectal cancer through the TSPO/VDAC pathway
Note: A: qRT-PCR for TSPO expression; B: qRT-PCR for changes of VDAC expression; C: CCK-8 for changes in the proliferation of HCT116; D: qRT-PCR for changes of P53 and Bcl-2; E: ELISA for changes of CEA and CA19-9. N=3, $^{*}P<0.05$, $^{**}P<0.01$

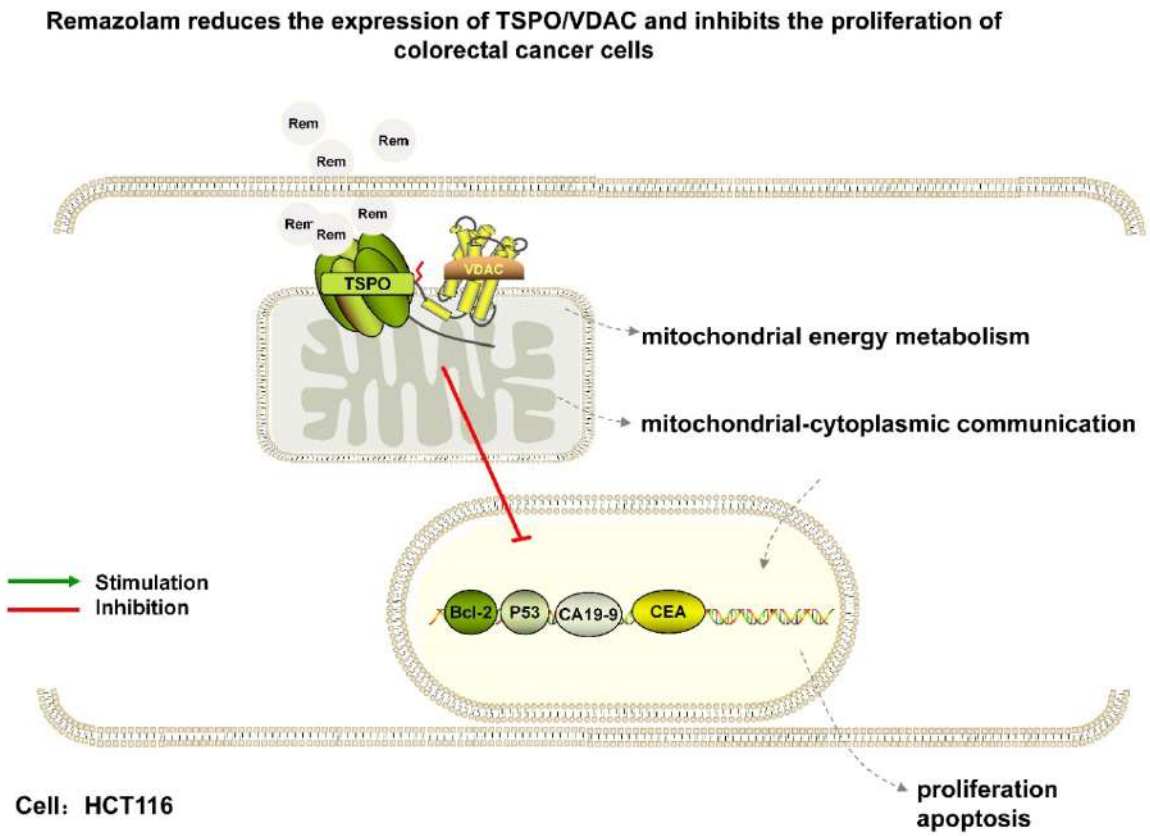

Fig. 6: Remimazolam reduces TSPO/VDAC expression and inhibits the proliferation of colorectal cancer cells

Discussion

We demonstrated that Rem affected mitochondrial energy metabolism and the mitochondria and cytosol dialogue through the TSPO/VDAC pathway, ultimately affected the proliferation and apoptosis of CRC cells (Fig. 6). CRC is the fourth leading cause of cancer death in 2019 (20,21), and the third leading cause of cancer death in 2021 (1). Patients with early and late-onset CRC failed to improve the quality of survival after chemotherapy regimen (22,23). Further investigation on effective medical intervention is needed (24). Targeted therapy is a new option for the treatment of CRC, which can successfully prolong the overall survival of CRC patients (25,26). This study found that Rem targeted CRC and inhibited the proliferation of CRC cells. Rem is a new short-acting BZD (27). Rem at 1 μg/mL inhibited the proliferation and promoted the apoptosis in HCT116 (Result 3), which is the first report, and this result expands the therapeutic range of Rem and BZD in the field of tumor treatment.

The pharmacological effect of BZD is required to bind with the peripheral BZD receptor (PBR) (8). TSPO is a PBR, which involves in the regulation of many cellular processes, including the inflammatory response, oxidative stress and mitochondrial homeostasis (28). This study found that TSPO was highly expressed in CRC (Results 1, 2). Previous studies also demonstrated that TSPO expression increased in cancer cells (29), which was consistent with this study. We found that that Rem not only inhibited TSPO expression (Result 5), but also reduced the expression of CRC markers, CEA and CA19-9, (Result 2). BZD is an antagonist of TSPO (30), whose application decreased TSPO expression (31), which

proved that Rem reduced TSPO expression and inhibited the proliferation of HCT116, as well as the expression of CRC-markers, CEA and CA19-9, and promoted the apoptosis of HCT116. TSPO, like VDAC, is also a mitochondrial protein (32,33). String online database analysis found that TSPO can interact with a variety of VDAC molecules, and the regulation of TSPO expression can affect VDAC expression in HCT116 (Result 4). Meanwhile, studies found that VDAC1 expression was higher in CRC and higher than that of surrounding healthy tissues (34), and silencing of VDAC1 expression by siRNA inhibited the proliferation of CRC cell lines (35). These findings are consistent with our findings, suggesting the expression pattern of the TSPO/VDAC pathway in CRC and the therapeutic potential of regulating the TSPO/VDAC pathway for CRC.

We found that the antiapoptotic protein Bcl-2 expression decreased, and the proapoptotic protein P53 expression increased after the inhibition of VDAC expression. However, Bcl-2 controls the permeability of mitochondrial outer membrane to cytochrome C and other apoptotic factors (36), so it can be speculated that the reduction of VDAC can reduce the dialogue between mitochondria and cytosol, change the energy metabolism of mitochondria, and ultimately affect the proliferation, apoptosis and the expression of CRC tumor markers, CEA and CA19-9. However, the use of Rem not only changed the expression of TSPO and VDAC, but also controlled the proliferation and apoptosis of CRC cells, and the activity of CRC tumor markers by inhibiting the TSPO/VDAC pathway.

Conclusion

Rem regulates TSPO/VDAC expression in CRC, affects the proliferation of CRC cells. Rem may affect the growth of CRC cells by regulating mitochondrial activity, and the detailed regulation of Rem regulating mitochondrial function remains to be further explored.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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