Brexpiprazole regulates EMT and metastasis through inhibition of SREBP1/SNAI1 signalling pathway in colorectal cancer cells

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Article

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Abstract

Objective: To investigate the role and mechanism of SREBP1/SNAI1 signalling pathway in the effect of brexpiprazole on EMT and metastasis of CRC.

Methods: The effects of different concentrations of brexpiprazole on the migration and invasion in vitro as well as the expression of proteins were examined by cell scratch, Transwell, Western blot, ELISA, immunofluorescence, dual luciferase promoter assay, transmission electron microscopy. A metastatic model of CRC in nude mice was established, Western blot, HE staining, and PET/CT were utilized to explore the effects of brexpiprazole on lung metastasis of CRC.

Results: Brexpiprazole significantly inhibited the migration and invasion of CRC, down-regulated the expression levels of SREBP1(m), Snail and MMP9, up-regulated E-Cad and ZO1 and decreased the secretion levels of ICAM-1 and VEGF in the supernatant of CRC. Western blot and dual luciferase assays showed that SREBP1 could directly govern on the expression level of SNAI1. In vivo experiments, on the other hand, showed that brexpiprazole significantly inhibited the formation of CRC lung metastases, suppressed the expression of SREBP1(m), Snail and MMP9, and up-regulated the expression of E-Cad and ZO1.

Conclusion: Brexpiprazole inhibited migration, invasion and metastasis of CRC by inhibiting the SREBP1/SNAI1 signalling pathway and down-regulating the expression of EMT-related factors.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of death in the world, making up approximately 10% of global cancer incidence and cancer mortality each year [1, 2]. Despite major advances in colorectal cancer treatment in recent years, recurrence and metastasis of CRC were still the leading causes of patient mortality today [2, 3]. A study have revealed that approximately 86% of patients with advanced CRC developed metastasis and died within 5 years [4]. Therefore, it is important in order to explore novel anticancer therapies for CRC.

Numerous studies have shown that EMT (epithelial-mesenchymal transition) was involved in the invasion and metastatic process of various tumours such as colorectal cancer, hepatocellular carcinoma, pancreatic cancer, etc. [5–8]. Down-regulation of the expression of the EMT marker factor E-Cad as well as elevation of the expression of Vimentin predicted the presence of lymph node metastasis, poor tumour differentiation, and a poor prognosis of patients with colorectal cancer, and the prevention or reversal of EMT in CRC may be a possible reduction of metastasis, recurrence, and drug resistance methods [5–7, 9]. In addition to the invasive and metastatic process, EMT was involved in other steps in the cancer process. For example, EMT can induced Snail1 and Snail2, the two main members of the gene Snail family, to express, and participated in lipid metabolism, apoptosis and immune escape of tumors [10].
Enhanced lipid synthesis and uptake contributed to tumour formation and progression, and dysregulation of sterol regulatory element binding proteins (SREBPs) played a central role in these processes and was an important feature of metabolic reprogramming in cancer\(^\text{[11–13]}\). Sterol regulatory element-binding protein1 (SREBP1), also known as sterol regulatory element-binding transcription factor 1 (SREBF1), was a major transcription factor regulating lipid metabolism and has become a biomarker for prognostic and drug efficacy monitoring in cancer patients\(^\text{[14–16]}\). It has been found that overexpression of SREBP1 significantly increased the oxygen consumption, filopodial formation, and migration and invasion ability of thyroid cancer cells, and silencing of SREBP1 significantly increased the expression level of E-Cad in oesophageal squamous cell carcinoma, and decreased the expression level of N-Cad, Vimentin, Snail, MMP9, and VEGF-c thereby inhibiting the proliferation of ESCC, migration and invasion\(^\text{[11, 12, 17]}\). In CRC, SREBP1 expression not only enhanced lipid metabolism and thus promoted the proliferation of tumour cells, but also activated the NF-κB pathway, increased MMP7 expression and promoted CRC invasion and metastasis\(^\text{[18]}\).

Brexpiprazole is a novel modulator of serotonin-dopamine activity. In July 2015, it was first approved in the United States\(^\text{[19–21]}\). It has recently been reported to function as an antitumour stem cell (CSCs), significantly inhibiting the growth of a wide range of tumour cells (lung cancer, pancreatic cancer, glioblastoma), decreasing the expression of Survivin, and reversing resistance to EGFR tyrosine kinase inhibitors, thus exerting anticancer effects\(^\text{[22–24]}\). Our group's pre-study has demonstrated that brexpiprazole can inhibit cell proliferation and lipid synthesis in CRC through the AMPK/SREBP1 signalling pathway\(^\text{[25]}\). However, whether brexpiprazole can also affect CRC EMT and inhibit its invasion and metastasis as well as the role of SREBP1 and Snail proteins family in it were not clearly understood.

In this paper, we explored the role of SREBP1/SNAI1 in the effects of brexpiprazole on EMT and metastasis of CRC by in vivo and in vitro experimental methods, which provided theoretical and experimental bases for the use of brexpiprazole in antitumour therapy.

1 Materials and methods

1.1 Cell lines and reagents

HCT116 and SW620 cells were obtained from Procell Life Science&Technology Co., Ltd. (Wuhan, China). Brexpiprazole was purchased from Bide Pharmatech Ltd. (Shanghai, China). Antibodies: E-Cadherin(CST, 3195S), SREBP1(Proteintech, 14088-1-AP), ZO1(Afinity, AF5145), Snail(CST, 3879T), MMP9(Huabio, ET1704-69), SNAI1(Proteintech, 13099-1-AP). Dual Luciferase Reporter Gene Assay Kit(RG027) was from Beyotime Biotechnology(Shanghai, China). hSREBP1 expression vector was provided by VectorBuilder Inc. (Guangzhou, China). Four SNAI1-siRNAs were designed and used with the assistance of Shanghai Gene Chemistry Co., Ltd. The sequences are as follows: sequence1, 5′-CCCACUCAGAUGUCAAGATT-3′; sequence2, 5′-CAGGACUCUAUCCAGAGTT-3′; sequence3, 5′-CUCCUCUACUUCAGUCUCUTT-3′; sequence4, 5′-AUGCUAUCUGGGACUCUGTT-3′.
1.2. Cell culture

Human colorectal cancer cell lines HCT116 and SW620 were cultured in McCoy’s5A and high sugar DMEM medium respectively, with the addition of 10% fetal bovine serum and 1% double antibody, incubated and grown in an incubator at 5% CO₂, 37°C.

1.3 Scratch wound healing experiment

Use the tip of 10μl pipette gun along the straightedge perpendicular to the horizontal line at the bottom of the well plate, gently scratched a vertical line in the cells, observed and took pictures under the inverted microscope. 48h later, observed and took pictures under the inverted microscope again, and experimented independently for three times.

1.4 Transwell invasion experiment

Adjust the cell density to 2.5×10⁵/ml, add 200μl of cell suspension to both the upper chamber of the well plate, and 600μl of culture medium containing 20% FBS to the lower chamber. 48h later, the chambers were stained and fixed, and the cells were observed and photographed under an inverted microscope.

1.5 Western blotting

Collect the cells of each group, adjust the cell number 1×10⁶/ml, centrifuge, discard the supernatant, add the lysis solution, after gel electrophoresis, transfer to the PVDF membrane, 5% skimmed milk powder close for 60min at room temperature, add the corresponding primary antibody, place in the refrigerator at 4°C for overnight incubation, and the secondary antibody was incubated at room temperature for 1h, and then developed and photographed in the chemiluminescence imaging system. The grey scale value of protein bands was analysed by Image J software.

1.6 Cell transfection

Spread the cells in 6-well plates and transfect them when the cell density was 80%. 200μl of serum-free medium was diluted with 3μg of DNA. 200μl of serum-free medium was diluted with 6μl of GP-transfect-Mate transfection reagent. Mix well and let stand at room temperature for 5 min. Add GP-transfect-Mate medium mixture dropwise to the DNA medium mixture, mix well and let stand at room temperature for 15 min, then transfect immediately. 400μl of transfection mixture was in addition to the wells and the final system was 2ml. Protein expression was detected after 48 h.

1.7 ELISA

Collect the supernatant of cell culture, centrifuge at 4°C for 20 min. Set up blank wells, standard wells, sample wells, each with 3 duplicate wells. Add the samples to the bottom of the enzyme-labelled well plate, seal the plate with sealing mould and place it in the oven at 37°C for 30 min. Wash the solution 5 times. Add 50μl of enzyme reagent to each well and incubate for 30 min. Add 50μl of colour developer A
and B successively and develop the colour at 37°C for 10 min, then add the termination solution to terminate the colour development. Read the OD value of each well at 450nm. The OD value of each well was read at 450nm, and the corresponding concentration was calculated according to the standard curve of OD value of the samples.

### 1.8 Immunofluorescence

Cells were inoculated into confocal dishes, set up 3 compound wells each for control and experimental groups, after fixation, permeabilisation, closure, primary antibody binding, secondary antibody binding, DAPI stained nuclei for 5min, observed and photographed under fluorescence microscope.

### 1.9 Dual luciferase promoter assay

Insert the promoter region of SNAI1 into a luciferase-expressed reporter gene vector to construct a reporter gene plasmid. Co-transfect cells with SREBP1 plasmid and reporter gene plasmid, measure the fluorescence value and analyse the data.

### 1.10 Animal experiments

The animal husbandry and experimental procedures were reviewed and approved by the Ethics Committee of North Sichuan Medical College (permit number: 2023095), in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

#### 1.10.1 Construct an animal model of colorectal cancer cell-making metastasis

Ten male Balb/c nude mice of 4-6 weeks old SPF grade were selected and acclimatised in a specific pathogen free (SPF) grade environment for one week. HCT116 cells in logarithmic growth phase were collected, the cell density was adjusted to $1 \times 10^6$/ml, and 100µl of cell suspension was injected into the tail vein of nude mice. They were randomly divided into control and experimental groups (n=5). The control group was given 5% gum arabic (100µl) by gavage once a day; the experimental group was given the same volume of epirubicin suspension by gavage once a day at a dose of 5mg/kg. 8 weeks later, the nude mice were anaesthetised with 1% pentobarbital and then killed by cervical dislocation, after which the lungs were opened and partly preserved at -80°C, and partly placed in 4% paraformaldehyde solution for subsequent HE staining.

#### 1.10.2 HE staining

Tumour tissue was fixed in 4% paraformaldehyde at room temperature for 24h, embedded in wax block and cut into 4µm thick slices. It was stained in hematoxylin and rinsed in water, stained in eosin stain for several seconds and rinsed in running water. The sections were sequentially dewaxed in ethanol solutions of varying concentrations, subsequently sealed, observed under a microscope, and documented through photography.
1.10.3 PET/CT scanning

Nude mice were anaesthetised by placing them in an anaesthesia box with a concentration of 2.5% isoflurane for 10 min, each was injected with 400µci of active 18F-FDG and placed in the prone position into the PET/CT scanning unit for detection and imaging.

1.11 Statistical analysis

All data were performed with GraphPad Prism software version 9.5.0, and the results obtained were expressed as mean±standard deviation (mean±SD). Multi-group data were tested by one-way ANOVA, and two-group data were tested by t-test. All experiments were repeated three times. The level of significance was set at p<0.05,*p<0.05,**p<0.01,***p<0.001,****P<0.0001.

2 Results

2.1 Brexpiprazole inhibits migration and invasion of colorectal cancer cells

We treated CRC cell lines (HCT116, SW620) with different concentrations of brexpiprazole (0, 5, 10, 20µM) for 24h. Scratch experiments showed that brexpiprazole significantly reduced the area of closed cell growth in a dose-dependent manner. Transwell experiments, on the other hand, showed that the perforation ability of CRC cells was also inhibited compared to the control group (Fig. 1A, B). We also observed the ultrastructure of CRC cells treated with brexpiprazole (20µM) by transmission electron microscopy and found that the microfilaments in the cells were significantly reduced (Fig. 1C red arrows at the red arrows, yellow arrows were the enlarged image of the area). It was evident that brexpiprazole inhibited the migration and invasion ability of CRC cells.

2.2 Brexpiprazole inhibits EMT in colorectal cancer cells

The expression of EMT-related proteins and related factors in HCT116 and SW620 cells were detected by Western blot and ELISA. The results showed that brexpiprazole down-regulated the expression of mesenchymal-associated proteins Snail and MMP9 and up-regulated the expression of epithelial-associated proteins ZO1, E-Cad in a dose-dependent manner (Figure 2A). The expression of ICAM-1, VEGF was gradually decreased with the increase of brexpiprazole drug concentration (Figure 2B).

2.3 Localisation and expression of SREBP1 and the key EMT protein E-Cad

In order to further explore the localisation and distribution of SREBP1 E-Cad in CRC cells, we used immunofluorescence to detect their expression in the cells. Immunofluorescence results showed that SREBP1 expression decreased after treatment with 20 µM of brexpiprazole and was distributed in the cytoplasm and nucleus while the expression of E-Cad increased and was mainly distributed in the cytosol (Figure 2C).

2.4 Brexpiprazole regulates EMT through SREBP1/SNAI1 and inhibits CRC metastasis
We constructed a colorectal cancer cell SREBP1 overexpression line and a SNAI1 low expression line to further clarify the role of SREBP1/SNAI1 in the metastatic process of colorectal cancer cells. Western blot was used to detect the expression of SREBP1 and the corresponding expression of EMT-related proteins. After overexpression of SREBP1, the mesenchymal-associated proteins Snail and MMP9 were elevated, the epithelial-associated proteins ZO1 and E-Cad were reduced (Figure 3A), and the expression of ICAM-1 and VEGF was elevated (Figure 3C). Meanwhile, we also examined the migration ability of the cells, and the scratch assay showed that overexpression of SREBP1 enhanced the in vitro migration ability of the cells (Figure 3B). Dual luciferase promoter assay showed that SREBP1 directly regulated SNAI1, which in turn affected the metastasis of CRC cells (Figure 3D). Western blot results showed that overexpression of SREBP1 resulted in an increase in the expression of SNAI1, and the knockdown of SNAI1 showed no significant change in the expression of SREBP1, suggesting that SREBP1 can directly regulate the metastasis of SNAI1, thus affecting the metastasis of colorectal cancer cells (Figure 3E).

### 2.5 Brexpiprazole inhibits CRC metastasis in vivo

In order to investigate the effect of brexpiprazole in vivo metastasis in animals, we constructed an animal model of colorectal cancer metastasis. Western blot detected the expression of SREBP1(m) and EMT-related factors in the fresh lung tissues of nude mice, and the results showed that compared with the control group, the expression of SREBP1(m), Snail, and MMP9 in the lung tissues of the experimental group was significantly decreased, and the expression of E-Cad, ZO1 expression was increased (Figure 4A). HE staining and PTE/CT scanning showed that the lung metastases of nude mice cancer cells were significantly reduced in the experimental group compared with the control group (Figure 4B, C, D).

### 3 Discussion

Brexiprazole is a second-generation antipsychotic drug for the treatment of major depressive disorder and schizophrenia with an enhanced medication safety profile \[23, 26, 27\]. The inhibitory effects of brexpiprazole on cell proliferation have been demonstrated in various tumor types, including non-small cell lung cancer, pancreatic cancer, and glioblastoma, but the mechanism of action was not fully understood \[19, 28, 29\]. In our previous studies, we utilized RNA transcriptome technology and bioinformatics to investigate the intricate association between brexpiprazole's anti-tumor effects and lipid metabolism of CRC and validated that brexpiprazole was able to effectively suppresses CRC cells proliferation through the AMPK/SREBP1 signaling pathway.

Numerous studies have shown that elevated SREBP1 expression is associated with metastasis, invasion, and poor prognosis in solid tumours such as endometrial, renal, hepatocellular, and breast cancers, and inhibition of SREBP1 may inhibit the growth, migration, and invasion of cancer cells \[7, 11, 15, 17, 18\]. Similarly, the present study demonstrated that brexpiprazole also can effectively suppresses the metastasis and invasion of CRC cells through the SREBP1 pathway, as evidenced by significant dose-dependent effects observed in both in vivo and in vitro experiments. However, the mechanism was not very clear. In further research, we found that overexpression of SREBP1 significantly increased the
migration and invasion ability of CRC cells, and the expression of SNAI1 was also increased, while knockdown of SNAI1 showed no significant change in the expression of SREBP1, and it was not difficult to conclude that SREBP1 can regulate the expression of SNAI1. The findings of this study suggested that brexpiprazole was likely to exert its effects by modulating the SREBP1/SNAI1 signaling pathway. To further clarify whether SREBP1 can directly act on SNAI1, it was found by dual luciferase experiments (Figure 3D) that SREBP1 can directly target SNAI1. Therefore, targeting SREBP1 would be an effective way to inhibit tumour proliferation and malignant progression [11, 14, 15, 16, 29].

Snail family proteins included Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc), which contained 4-6 C2H2-type zinc fingers at the C-terminal end, mainly binding to E-box sequences in the promoters of target genes, and a conserved SNAG domain at the N-terminal end, which was essential for binding to transcriptional co-repressor complexes [30, 31]. Clinical analyses have confirmed that elevated Snail expression correlates with tumour size, lymph node metastasis, distant metastasis, clinical stage grading, and poor prognosis in CRC patients [32-34]. Studies have shown that Snail1 significantly inhibited E-Cad, regulated the expression of matrix metalloproteinases MMP2 and MMP9, and enhanced the invasive ability of colorectal cancer cells, giving them cancer stem cell-like characteristics and promoting drug resistance, tumour recurrence and metastasis [30-32]. In our study, brexpiprazole significantly exerted a down-regulatory effect on the expression levels of E-Cad, MMP2, and MMP7 in colorectal cancer cells as well as Snail expression in a concentration-dependent manner. In addition, after transfection of SREBP1, the migration ability of tumour cells was significantly increased, and the expression of Snail was also increased, which provided the idea that brexpiprazole could regulate EMT and metastasis of CRC cells through SREBP1/SNAI1 pathway. Unfortunately, we only verified the regulatory relationship between SREBP1 and Snail1 at the gene level through dual luciferase, and the evidence of action each other at the protein level was still insufficient. The relationship between SREBP1 and SNAI1 will be further validated in subsequent studies through the application of immunocoprecipitation or mass spectrometry techniques.

4 Conclusion

In summary, brexpiprazole regulated the expression of EMT-related factors in colorectal cancer cells by inhibiting the SREBP1/SNAI1 signalling pathway, thus down-regulating their invasion and metastasis, which suggested that SREBP1 was expected to be a new target for the prevention and treatment of invasion and metastasis of colorectal cancer, and at the same time, it also provided certain theoretical and experimental bases for the use of brexpiprazole in preventing and controlling colorectal cancer.

Declarations

Author Contribution Statement

Xiaojie Liu: Data analysis and Writing

Wenjun Xia: Software
Jingyi He: Investigation
Zhiyang Xia: Formal analysis
Wei Chen: Review and Editing

**Data availability statement**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

**Competing Interests Statement**

The author(s) declare no competing interests.

**Statement**

The study is reported in accordance with ARRIVE guidelines.

**References**


**Figures**
Figure 1

Inhibition of in vitro migration and invasion ability of colorectal cancer by brexpiprazole.

A-B. Scratch assay, Transwell assay detected the effect of brexpiprazole to inhibit the in vitro migration and invasion ability of colorectal cancer cells HCT116, SW620. C. Transmission electron microscopy.
observed the effect of 20µM ipipiprazole on cellular microfilaments.
*p<0.05,**p<0.01,***p<0.001,****p<0.0001.

Figure 2
Brexpiprazole inhibited the localised expression of EMT-related proteins as well as SREBP1 and E-Cad in CRC.
A. Western blot detected the effects of different concentrations of brexpiprazole on the expression levels of SREBP1 (m), Snail, E-Cad, ZO1, MMP9 proteins in CRC cells HCT116, SW620. B. ELISA detected the expression levels of ICAM-1, VEGF in the supernatants of the cells. C. Immunofluorescence detected the localised expression of SREBP1, E-Cad in the cells after 20µM SREBP1, E-Cad localised expression after brexpiprazole treatment of cells. *p<0.05, **p<0.01, ***p<0.001.
Brexpiprazole regulated EMT-related proteins through SREBP1/SNAI1, thereby affecting colorectal cancer metastasis.

A-B. After overexpression of SREBP1, Western blot detected the protein expression levels of SREBP1(m), Snail, E-Cad, ZO1, MMP9 in colorectal cancer cells HCT116 and SW620 as well as scratch assay to detect in vitro migratory ability. C. ELISA was performed to examine the expression levels of ICAM-1 and VEGF in the supernatants of cells. D-E. Dual luciferase promoter assay and Western blot demonstrated that SREBP1 directly regulated SNAI1. *p<0.05,**p<0.01,***p<0.001,****P<0.0001.
Figure 4

Brexpiprazole reduced the metastatic ability of colorectal cancer cells in vivo.

A. Effects of brexpiprazole on the expression levels of SREBP1(m), Snail, E-Cad, ZO1, MMP9 proteins in colorectal cancer in vivo.

B. PET/CT scanning for detecting metastasis in the lungs of nude mice.

C-D.
Arrowed portions were metastatic foci formed by metastasis of CRC, and there were basically no metastatic foci in the experimental group. *p<0.05,**p<0.01.

**Supplementary Files**

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