Expression of sphingosine-1-phosphate receptor 2 is correlated with migration and invasion of human colon cancer cells: A preliminary clinical study

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Abstract. Sphingosine-1-phosphate (S1P) is a bioactive phospholipid that serves as a potent mediator of cell proliferation, differentiation and apoptosis by binding to S1P receptors (S1PRs). S1P signalling is involved in the pathogenesis of numerous types of disease, including cancer. To the best of our knowledge, however, little is known about the expression patterns of S1PRs and their role in human colorectal cancer (CRC) cell migration and invasion. The aim of the present study was to investigate the role of S1P signalling in the metastasis of colon cancer cells and the expression of S1PRs in patients with CRC. The protein and mRNA expression levels of S1PRs and sphingosine kinases (SPHKs) in 55 patients with CRC were detected by western blotting (WB), immunohistochemical (IHC) analysis and reverse transcription-quantitative PCR. The levels of S1P in serum from patients and healthy individuals were quantified by ELISA. S1PRs antagonists JTE013, FTY720 and S1PR2-small interfering (si)RNA were used to determine the role of S1PR2 in human CRC LOVO and SW480 cell lines. Migration and invasion assays were performed for functional analysis. The levels of S1P in serum were significantly increased in patients with CRC compared with healthy individuals. The relative mRNA expression levels of S1PR2 were significantly downregulated in tumour compared with normal tissue, whereas S1PR1 and SPHK1 were upregulated. WB showed that 58% (32/55 cases) of patients presented downregulated S1PR2 protein expression. IHC analysis indicated that expression of S1PR2 was lower in tumour than in normal tissue in 65.5% (36/55 cases) of patients. Exogenous addition of S1P promoted migration and invasion in the different cell types. S1P stimulated the migration and invasion of SW480 cells. The inhibition of S1PR2 by JTE013 or S1PR2-siRNA significantly promoted the migration and invasion of SW480 cells, while FTY720 reversed these effects. The present study indicated that expression levels of S1PRs, particularly S1PR2, were associated with migration and invasion of CRC cells. The present findings revealed a novel mechanism by which S1P inhibited tumour cell migration and invasion via a S1PR2-dependent pathway, suggesting that S1PR2 may be a therapeutic target for treatment of colon cancer.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the second leading cause of cancer-associated mortality worldwide. In 2017, 1,688,780 new cancer cases and 600,920 cancer-associated deaths were projected to occur in the United States (1). CRC represents 8.4% of all novel cancer cases and has emerged as a public health concern due to its high morbidity and mortality rates. According to estimates from the National Cancer Institute, there are 145,600 novel

cases of colon and rectal cancer every year, and nearly 51,020 Americans die per year as a result of CRC. Despite this, the 5-year relative survival rate of CRC has increased from 48.6 to 64.4% over past decades, potentially due to increased awareness of risk factors and widespread implementation of endoscopic screening (1). Researchers are working to advance understanding of how to prevent, detect and treat CRC. To the best of our knowledge, however, the precise aetiology of CRC and the underlying molecular mechanism remain unclear. Previous studies have shown that disturbance of sphingosine-1-phosphate (S1P) signalling and metabolism is associated with the inflammatory response and numerous types of disease, including the development and progression of cancer (2-10). S1P is a membrane-derived bioactive phospholipid synthesized by sphingosine kinases (SPHKs) 1 and 2 from sphingosine and degraded by S1P lyase in mammalian cells (11). S1P is exported from cells and serves as a signal transducer via G protein-coupled S1P receptors (S1PRs) 1-5 or activates intracellular targets directly to regulate cell homeostasis and biological functions, including cell proliferation, migration, invasion and control of immune cell trafficking and angiogenesis (12). The aforementioned steps, which make up the S1P axis, might protect patients from colon cancer (13). Data from human tumour cell lines and tissue show that aberrant expression of S1P-regulating enzymes and receptors is a key initiating event for malignant transformation and colon cancer progression (14,15).

S1PR1, S1PR2 and S1PR3 are ubiquitously expressed S1PRs that mediate diverse functions of S1P in multiple types of cell, including regulating proliferation and migration, whereas S1PR4 and S1PR5 are restricted to the immune and nervous systems, respectively (16). S1P binds to different subtypes of S1PRs to elicit biological responses (9). These receptors are exclusively coupled to heterotrimeric G proteins and Rho or Rac to control various effector systems, including adenylate cyclase, phospholipases C and D and extracellular-regulated p38 mitogen-activated protein, c-Jun N-terminal and non-receptor tyrosine kinase (17-19). S1PR1 and S1PR3 mediate potent stimulation of migration and invasion of tumour cells by inducing ERK activation, whereas S1PR2 exhibits an inhibitory effect via Rho activation and Rac inhibition (20-23). Therefore, the role of S1PRs in cancer remains controversial.

Studies have shown expression or mutation of S1PRs in cancer (24-27). Yoshida *et al* (24) demonstrated that down-regulated expression of S1PR1 increases proliferative activity to enhance the malignancy of tumours, resulting in poor survival in patients with glioblastoma. Kothapalli *et al* (25) reported that the S1P5 gene is overexpressed in large granular lymphocyte leukaemia and may protect against apoptosis of these cancer cells. Flori *et al* (27) suggested that S1PR2 is a novel tumour suppressor and survival prognosticator in activated B cell-like diffuse large B-cell lymphoma. To the best of our knowledge, however, little is known about the biological function of S1PR2 and its potential role in CRC. To determine the molecular pathogenesis of CRC, the present study performed functional analysis of sphingolipids, focusing on the role of S1PR2 in colon cancer cell line proliferation and migration. Expression of S1P, its target receptor S1PR1-5 and its synthetase SPHKs in human colorectal carcinoma tissue was assessed and the association between expression of S1PR2 and clinical variables was determined. The study findings may provide new insight into the development of new anti-CRC drugs through regulation of the S1P/S1PR2 axis.

Materials and methods

Human specimens. Fresh colon cancer (n=55) and matched non-tumourous colon tissue (NT) (n=55) were collected following surgical resection at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) from 2010 to 2015. The NT tissue was obtained at the resection margin, located at a distance of >5 cm away from the primary tumour. The mean age at diagnosis was 64.00±11.91 years. Plasma was collected from patients with colon cancer (n=55) and sex- and age-matched healthy donors (n=55; mean age, 74.51±8.42 years; 25 male and 20 female). Tissues were washed in ice-cold PBS and a portion of tissues were formalin-fixed and paraffin-embedded. The rest of the tissue and plasma samples were stored at -40˚C. All patients had a histological diagnosis of colon cancer and none had received therapy. All specimens were obtained with verbal consent from the patients and healthy donors, and the study was approved by the Institutional Ethics Committee of Nanjing Medical University (approval no. 2016-SR-217).

Immunohistochemical (IHC) staining. As 5 of the specimens were not large enough, 50 cases were included in the IHC assay. The tissues were fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min and embedded into paraffin. Tissue paraffin blocks were serially sectioned at 4 μm. Sections were deparaffinized at 60˚C for 30 min and washed in xylene, and rehydrated in a descending alcohol series. Tissue sections were placed in a repair box filled with EDTA antigen repair buffer (pH 9.0) (Wuhan Goodbio Technology, Co., Ltd.; cat. no. G12036), and antigen retrieval was performed by microwaving for 15 min, endogenous peroxidase was blocked at room temperature with 3% hydrogen peroxide in methanol for 25 min and tissue was blocked with normal rabbit serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 16120107; 1:10) for 30 min at room temperature. CRC tissues were incubated with anti-S1PR2 primary antibody (Santa Cruz Biotechnology, Inc.; cat. no. sc-365589; 1:500) overnight at 4˚C. Sections were washed with PBS and incubated with an HRP-conjugated secondary antibody (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A16160; 1:1,000) at room temperature for 50 min. After being washed with PBS, the target protein was stained with 1% 3,3'-diaminobenzidine(DAKO; Shanghai Li Min Industrial Co., Ltd.; cat. no. K5007) at room temperature for 5 min, and cell nuclei were counterstained blue with haematoxylin at room temperature for 3 min. The slides were scanned using the Pannoramic Midi II histological scanner 1.18.1 (3DHISTECH Ltd.). Next, using the Pannoramic Viewer Program 1.15.3 (3DHISTECH Ltd.), representative areas were selected. A light microscope (IX73; Olympus) was used for imaging at x100 and x200 magnification. The staining score for tumour and adjacent normal tissue (0, negative; 1+, weak; 2+, moderate and 3+, strong) was recorded separately. The estimated proportion of positive tumour cells was calculated as a percentage. To assess the mean degree of staining within

a tumour, three representative regions were analysed and ≥100 tumour cells were assessed. S1PR2 expression was assessed by histochemistry (H)-score system as follows: H-score=∑(IxPi), where I=intensity of staining and Pi=percentage of stained tumour cells, producing a score ranging from 0 to 300. The scoring was independently assessed by two investigators who were blinded to the patient characteristics and outcomes.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from tissue with an RNA Extraction kit (Takara Biotechnology Co., Ltd.; cat. no. 9767) according to the manufacturer's protocol. Total RNA was quantified using a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific, Inc.). Only RNA with a 260/280 ratio of 1.8-2.0 was used for cDNA synthesis and reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Biotechnology Co., Ltd.; cat. no. RR036A) at 37˚C for 15 min. mRNA levels of target genes were detected by qPCR using SYBR premix Ex Taq™ (Takara Biotechnology Co., Ltd.; cat. no. 638319) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec and extension at 60˚C for 1 min. Following normalization using β-actin as an internal control, the data were analysed using the ΔΔCq method and expressed as target gene/internal ratio [$2^{-\Delta Cq(\text{target gene-internal})}$] (28). The primers were as follows: S1PR1 forward, 5'-TCCTCGCCATCGCCATTG-3' and reverse, 5'-GAGAGCAGAAGCAGAGTGAAG-3'; S1PR2 forward, 5'-CAAGGTCCAGGAACACTATA-3'and reverse, 5'-AACAGAGGATGACGATGAA-3'; S1PR3 forward, 5'-CTACGCACGCATCTACTTCC-3' and reverse, 5'-CACGCTCACCACAATCACC-3'; S1PR4 forward, 5'-GCTGAAGACGGTGCTGATG-3' and reverse, 5'-CTGCTGCGGAAGGAGTAG-3'; S1PR5 forward, 5'-CTTCCTGCTGCTGTTGCTC-3' and reverse, 5'-GCCACTCGGGTCTCTGC-3'; SPHK1 forward, 5'-TGTGTAGCCTCCCAGCAG-3' and reverse, 5'-CCCAGACGCCGATACTTC-3'; SPHK2 forward, 5'-ACTGCCCTCACCTGTCTG-3' and reverse, 5'-TTCTGTCGTTCTGTCTGGATG-3' and β-actin forward, 5'-TGACGTGGACATCCGCAAAG-3' and reverse, 5'-CTGGAAGGTGGACAGCGAGG'.

Quantification of S1P. Quantification of S1P from serum was performed by ELISA using a Human S1P Assay kit (Nanjing SenBeiJia Biological Technology Co., Ltd.; cat. no. SBJ-H2060-96T) according to the manufacturer's instructions.

Cell culture and treatment. CRC SW480 and LOVO cell lines, purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, were maintained in DMEM supplemented with 10% foetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA). All cells were cultured at 37˚C with 5% CO_2. Cells were grown in 100-mm dishes and incubated at 37˚C in DMEM for 24 h. The small interfering RNA (siRNA) oligonucleotide duplexes (si-S1PR2: 5'-CCUUCGUAGCCAAUACCUUTT-3'; negative control, 5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUUCGGAGAATT-3'; Shanghai GenePharma Co., Ltd.) were transfected into cells using Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.). S1PR2 siRNA (120 pmol) and transfection reagent (6 μl) were mixed in 300 μl serum-free DMEM, left to stand for 5 min and then mixed again. Following incubation at room temperature for 20 min, the mix was added to serum-starved cells and incubated at 37˚C for 4 h. CRC cells were plated the day before transfection at 400,000 cells/well in 6-well plates in complete growth medium (Gibco; Thermo Fisher Scientific, Inc.). S1P, JTE013 and FTY720 were purchased from Cayman Chemical Company and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). Cells were pre-treated at 37˚C with S1PR2 antagonist JTE013 (5 μM), S1PR1,3,4,5 antagonist FTY720 (5 μM) or DMSO (1%) for 1 h before stimulation in the presence or absence of S1P (1 μM) at 37˚C for 1 h. DMSO was applied as vehicle. Cells were harvested 24 h later for migration and invasion assay.

Cell viability assay. Human CRC SW480 cells ($5x10^3$ cells/well) were seeded into 96-well plates, allowed to adhere and serum-starved overnight at 37˚C in 95% air and 5% CO_2. Next, cells were transfected with S1PR2-specific siRNA and treated with S1P (1 μM) or DMSO. Following 24 h incubation at 37˚C, cells were stained with sterile MTT for 4 h at 37˚C, the supernatant was collected by centrifugation at 13,225 x g for 2 min at 4˚C and removed, and the crystals formed were dissolved using DMSO. The absorbance at 490 nm was examined.

Wound healing assay. SW480 or LOVO cells were plated at a density of $5x10^5$ cells/well in 6-well culture dishes and allowed to form a monolayer (70-90% confluence). Following serum-starvation for 24 h, the cells were scratched with a sterile pipette tip (200-μl), washed with PBS to remove floating and detached cells and photographed (time, 0 h) under the 10x objective of an Olympus 1X71 light microscope (Olympus Corporation). Cells were pre-treated with JTE-013 (5 μM), FTY720 (5 μM) or DMSO for 1 h and treated with S1P (1 μM) or DMSO. After 24 h, the wound area was. The assay was performed three times. ImageJ v1.8.0 software (National Institutes of Health) was used to analyse the cell-free areas in images. A total of 6-8 horizontal lines were drawn on each image and the width was calculated.

Transwell cell invasion assay. After being starved overnight, SW480 cells ($6x10^3$ cells/well) were seeded in a Matrigel-coated upper polycarbonate chamber (8-μm pore size; BD Biocoat Matrigel Invasion Chamber). The chambers were precoated with 20% Matrigel for 30 min at 37˚C. The upper chamber was filled with serum-free DMEM and the lower chamber was filled with medium containing 10% FBS (Gibco-BRL, Rockville, MD, USA). Cells were pre-treated with JTE-013 (5 μM), FTY720 or vehicle DMSO for 1 h, then treated with S1P (1 μM) or DMSO and incubated at 37˚C in 95% air and 5% CO_2. After 24 h, non-invasive cells were washed off twice with PBS. The cells that had migrated to the lower surface were fixed for 30 min with 3.7% paraformaldehyde at 25˚C and stained for 20 min using 0.1% crystal violet solution at 25˚C, and then observed and images captured at x200 magnification on a light microscope. For each replicate (n=3), migrated

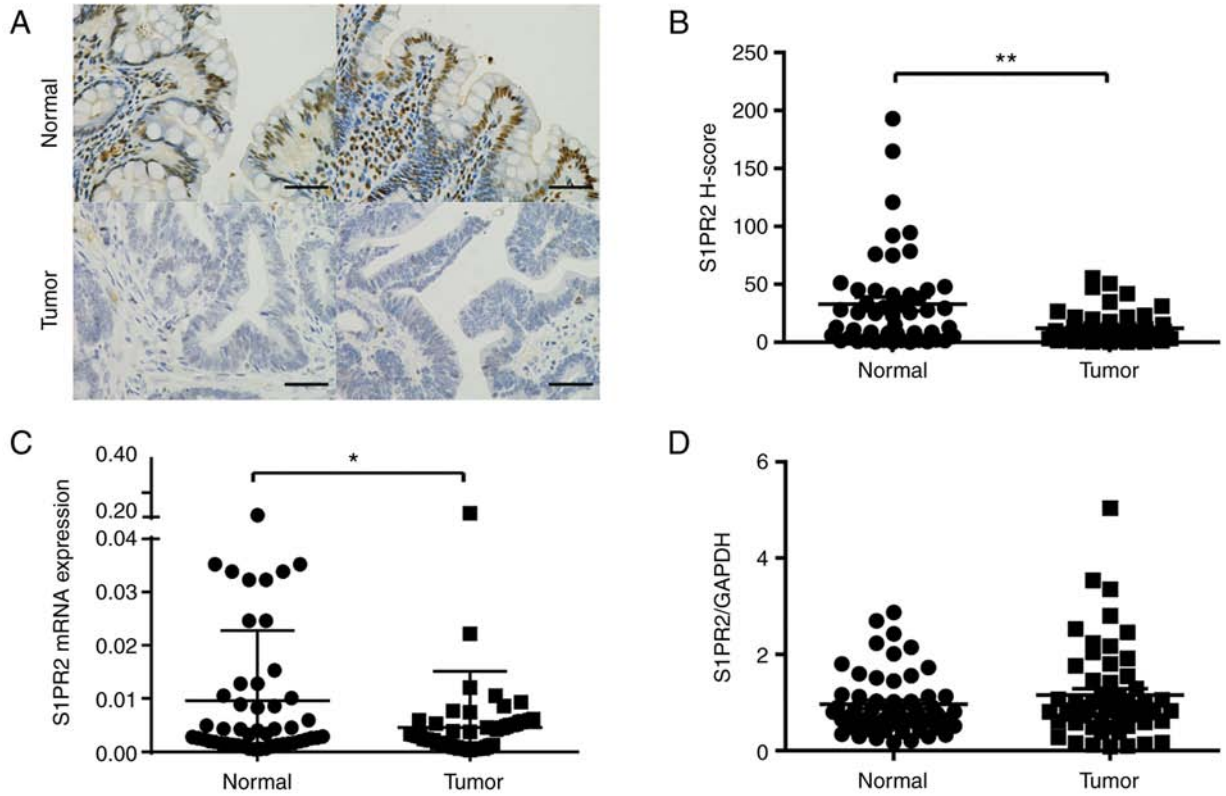

Figure 1. S1PR2 expression in colon tissue of patients with CRC. (A) Representative images of S1PR2 staining in human CRC tissues. Scale bar, 50 μm. (B) Expression of S1PR2 determined by H-score in the colon tissue of patients with CRC (n=50; **P<0.01; Wilcoxon's signed-rank test). (C) Relative mRNA expression levels of S1PR2 in tumour and adjacent non-tumourous tissue from patients with CRC determined by reverse-transcription quantitative PCR. Data were normalized using β-actin as an internal control. *P<0.05 (n=55; P=0.0114; Wilcoxon's signed-rank test). (D) Protein expression of S1PR2 relative to GAPDH in tumour and normal colon tissue from patients with CRC (n=55; P=0.45; Wilcoxon's signed-rank test). Error bars=SEM. S1PR, sphingosine-1-phosphate receptor; CRC, colorectal cancer; H, histochemistry.

cells were manually counted and expressed as the average cell number from 4 random fields. The cell number indicated the cell migration capability and invasiveness.

Western blotting. Tissues samples were harvested and lysed in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium cholate, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM HEPES, 3 mM MgCl, 1 mM PMSF, 20 mM b-glycerophosphate, 1 mM NaF and 1 mM sodium orthovanadate; pH 7.4) and sonicated (20 kHz; 0˚C; 30 sec). Following centrifugation at 13,225 x g for 20 min at 4˚C, protein concentrations were assayed using Bradford protein assay reagent (Bio-Rad Laboratories, Inc.) with BSA as a standard. Total protein (30 μg) was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). The membranes were blocked with 5% non-fat milk in TBST [10 mM Tris (pH 7.4), 100 mM NaCl and 0.5% Tween 20] for 1 h at room temperature and incubated with the primary antibody S1PR2 (Santa Cruz Biotechnology, Inc.; cat. no. sc-365589; 1:1,000). Protein bands were normalized to GAPDH and detected with anti-GAPDH (Santa Cruz Biotechnology, Inc.; cat. no. sc-47724; 1:1,000). Immunoreactive bands were detected using rabbit anti-mouse IgG HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.; cat. no. sc-358914; 1:5,000) and SuperSignal Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The densities of bands were analysed using Image Lab software.

Statistical analysis. Experiments were repeated ≥3 times and data are presented as the mean and standard error of the mean (SEM). Statistical analysis for multiple comparisons was performed by one-way ANOVA followed by post hoc Bonferroni's correction. The differences in levels of S1P between the CRC group and healthy donor group were analysed using an unpaired Student's t-test. S1PR2 expression in tumour and matched normal tissues were compared using Wilcoxon's signed-rank test. P<0.05 was considered to indicate a statistically significant difference. All statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc.) software.

Results

Expression of S1PR2 is downregulated in human CRC. To test whether S1PR2 serves a role in human CRC, IHC analysis of S1PR2 in human colon tissue was performed. As 5 of the specimens were not large enough, 50 cases were included in the IHC assay. CRC specimens (n=50) had notably lower levels of S1PR2 than normal human colon tissue (n=50), which exhibited strong S1PR2-positive staining in colon cells (Fig. 1A). IHC staining was performed to evaluate expression of S1PR2 in tumour and adjacent normal tissue of patients with CRC using H-score as a continuous variable. Mean H-score was higher in normal compared with tumour tissue (33.11 vs. 12.23; P<0.001). S1PR2 expression in human CRC tissue was significantly lower

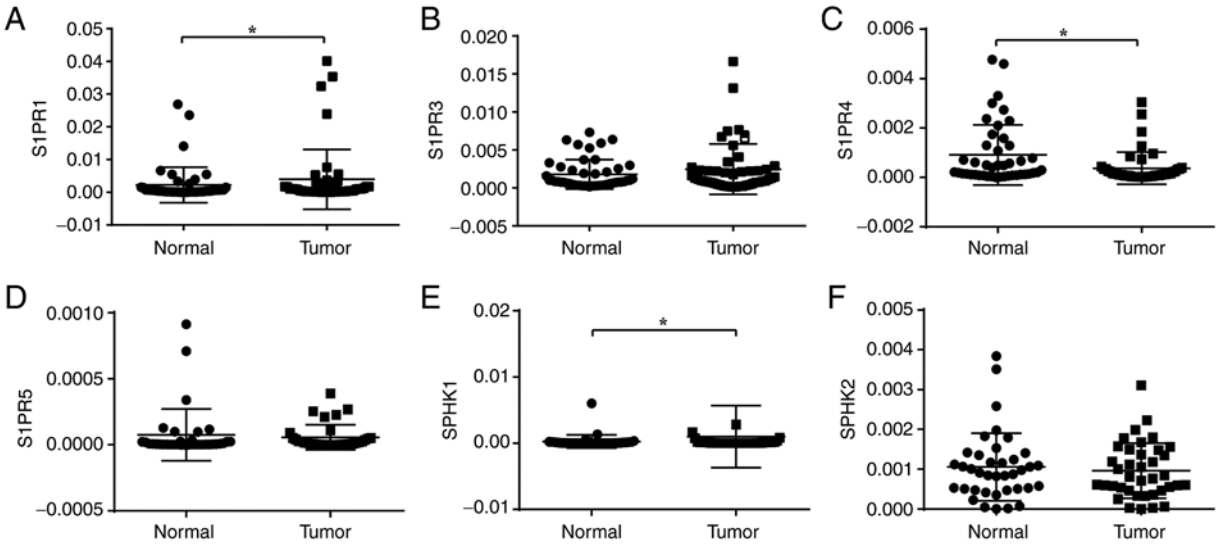

Figure 2. Relative mRNA expression levels of S1PR and sphingolipid metabolism enzymes in human colorectal cancer tissue. Expression of S1PR (A) 1, (B) 3, (C) 4 and (D) 5 and SPHK (E) 1 and (F) 2 in tumour and adjacent non-tumourous tissue was determined by reverse transcription-quantitative PCR (n=55; Wilcoxon's signed-rank test). Data were normalized using β-actin as an internal control. *P<0.05. S1PR, S1PR, sphingosine-1-phosphate receptor; SPHK, sphingosine kinase.

in comparison with that in paired normal colon tissue (n=50; Fig. 1B). Next, expression levels of S1PR2 mRNA in tumour and adjacent non-tumourous tissue of patients with CRC were assessed using RT-qPCR (n=55). S1PR2 was significantly downregulated in colon cancer compared with non-tumourous tissue, with a log2 expression ratio of -3.46 (tumour vs. normal; Fig. 1C). The protein level of S1PR2 in colon tissue was determined using western blotting (Fig. 1D). Among patients with CRC (n=55), downregulated S1PR2 was observed in 32 (58%) cases; however; this was not significantly different (P=0.45; Wilcoxon's signed-rank test). The mean ratio of S1PR2/GAPDH was slightly higher in tumour compared with normal tissue (1.15 vs. 0.96) but this was not significantly different. Taken together, these data showed that S1PR2 was downregulated in CRC cells and low S1PR2 expression may serve a role in the development of colon cancer.

Expression of S1PRs and SPHKs in human CRC. To confirm the role of the S1P axis in colon cancer, mRNA expression levels of S1PR1, S1PR3, S1PR4 and S1PR5 were measured in the tumour and adjacent non-tumourous tissue of patients with CRC using RT-qPCR (Fig. 2A-D). S1PR1 and S1PR3 were highly expressed S1PR subtypes in human colon tissue, whereas S1P4 and S1P5 were barely detectable. Transcript levels for S1PR1 gene were significantly higher in tumour than in normal tissue, while those of S1PR4 gene were lower (P<0.05). Transcripts of S1PR3 gene were higher in tumour tissue but there was no significant difference (P=0.0748). The results were consistent with previous studies that suggested that S1PR1 and S1PR3 are upregulated in numerous types of cancer (27). mRNA expression levels of SPHK1 and SPHK2 in colon tissue were measured (Fig. 2E and F). SPHK1 gene expression was significantly upregulated in tumour tissue, which is consistent with previous data (29). These results demonstrated variation in expression of S1PR and sphingolipid metabolism enzymes in colon cancer.

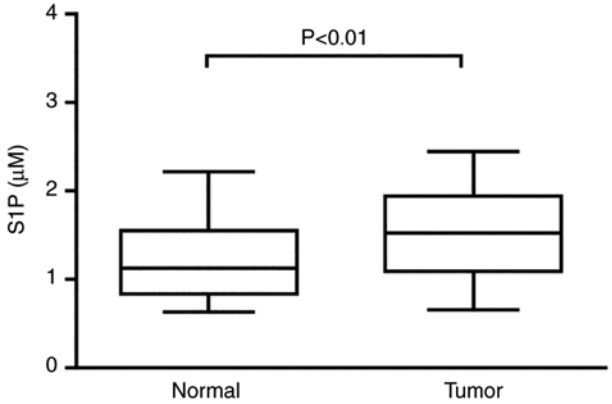

Figure 3. S1P levels in healthy donors and patients with CRC. Detection of S1P in serum by ELISA collected from healthy donors (n=55) and patients with CRC (n=55). Error bars=SEM. S1P, sphingosine-1-phosphate; CRC, colorectal cancer.

Level of S1P is higher in patients with CRC. Activation of SPHK1 leads to synthesis and secretion of S1P, which has been shown to influence cellular function via interactions with specific receptors, including S1PR2 (30). Expression of S1P in the peripheral blood of patients with CRC may be altered via the SPHK/S1P/S1PR signalling pathway. Therefore, S1P levels were measured in serum from patients with CRC (n=55) and healthy individuals (n=55) using ELISA. S1P level were higher in patients with CRC (Fig. 3).

Migration of human colon cell lines is increased by inhibition of S1PR2. S1P has been shown to regulate cellular physiological functions, including proliferation and migration, in different types of cancer (4). To determine the effect of exogenous S1P on the colon cancer cell lines SW480 and LOVO *in vitro*, wound healing assay was performed to investigate the role of S1P in colon cancer cell migration. LOVO cells treated with 1 μM S1P showed a significant increase

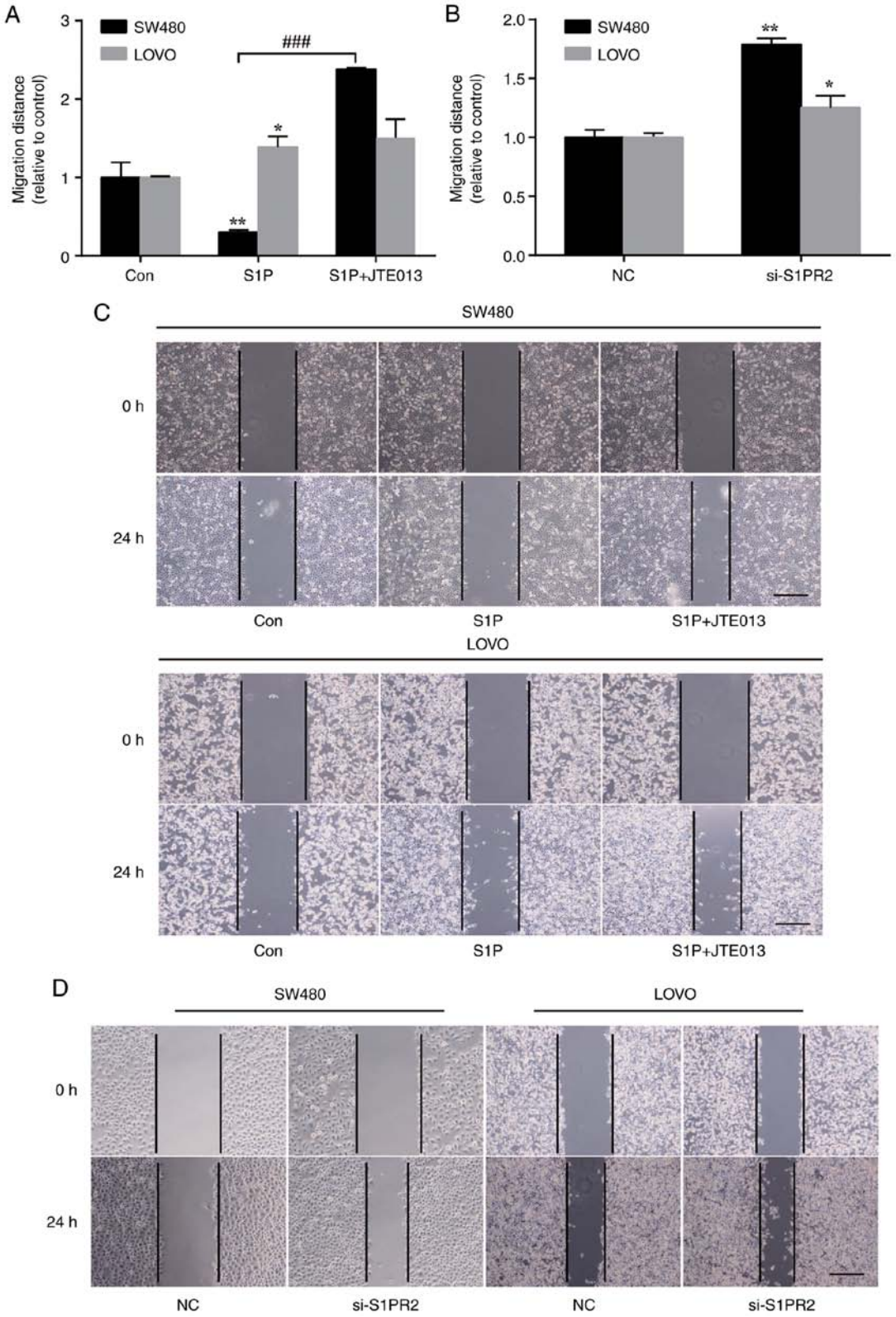

Figure 4. Effect of S1PR2 on cell migration. (A) Wound healing assay was performed in the presence or absence of 1 μM S1P and 5 μM JTE013. *P<0.05, **P<0.01, Con vs. S1P; $^{###}$P<0.001, S1P vs. S1P + JTE013. (B) Wound healing assays were performed in cells transfected with S1PR2-specific siRNA or control. n=3. *P<0.05, **P<0.01, NC vs. si-S1PR2. (C) Representative images of the wound healing assay. Wound healing assay was performed in the presence or absence of 1 μM S1P and 5 μM JTE013. Scale bar, 200 μm. (D) Representative images of the wound healing assay. Wound healing assays were performed in cells transfected with S1PR2-specific siRNA or control. Scale bar, 200 μm. S1PR, S1PR, sphingosine-1-phosphate receptor; Con, control; NC, negative control.

in migration distance, whereas that of SW480 cells treated with 1 μM S1P was significantly inhibited (Fig. 4A). JTE013, a specific S1PR2 antagonist, further promoted migration of SW480 and LOVO cells stimulated by S1P (Fig. 4A and C). To confirm the role of S1PR2 in cell migration, siRNA was used to specifically silence S1PR2 expression. Relative to the

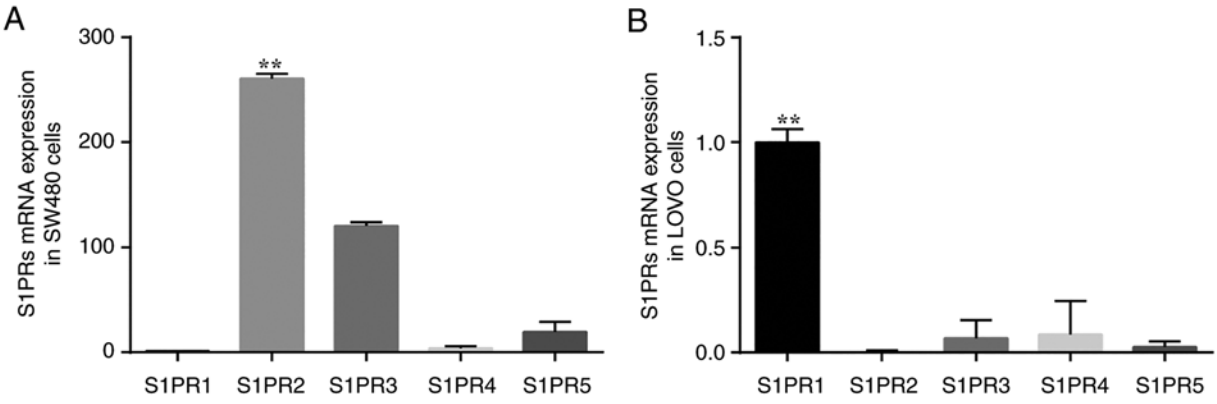

Figure 5. S1PR expression in human colorectal cancer cell lines. The expression levels of S1PRs in (A) SW480 and (B) LOVO cells were determined by reverse transcription-quantitative PCR (n=3). Expression levels are normalized to β-actin. **P<0.01, S1PR1 vs. S1PR2. S1PR, S1PR, sphingosine-1-phosphate receptor.

vehicle control, knockdown of S1PR2 by siRNA significantly promoted migration of SW480 and LOVO cells, which is consistent with the aforementioned results (Fig. 4B and D). Inhibition of S1PR2 by JTE013 or S1PR2-siRNA significantly promoted migration of SW480 and LOVO cells. These data indicated that expression of S1PR2 may inhibit the migration of colon cancer cells. Expression levels of S1PR in SW480 and LOVO cells were determined. Transcripts for S1PR genes were expressed at varying levels in colon cancer cells (Fig. 5). The mRNA expression levels of S1PR2 and S1PR3 were high in SW480 cells (Fig. 5A), whereas S1PR2 was expressed only at low levels in LOVO cells (Fig. 5B). Therefore, the effect of S1P on migration of different colon cancer cell lines may depend on S1PR subtypes expressed on the cell surface.

S1PR2 mediates the effect of S1P on migration and invasion of SW480 cells. To determine the role of S1PR2 in the migration and invasion of SW480 cells, wound healing and Transwell assays were performed using SW480 cells pre-treated in the presence or absence of JTE-013 and FTY720 for 1 h before S1P stimulation. There was no significant difference between the S1P and the control group with regard to the wound healing of SW480 cells (Fig. 6A). Treatment with 5 μM JTE-013 increased SW480 cell migration but this was not significant (Fig. 6A and C). Quantification of cell migration width confirmed a significant increase in cell migration following treatment with JTE013 + S1P compared with JTE013-alone (Fig. 6A; P<0.05). Co-treatment with S1P and 5 μM FTY720, an inhibitor of other S1PRs (S1PR1, 3, 4 and 5), significantly attenuated migration compared with the JTE013 + S1P group (Fig. 6A; P<0.01). S1P significantly enhanced invasion of SW480 cells in Transwell assay compared with the control group (Fig. 6B; P<0.01). Pre-treatment with JTE013 further promoted SW480 cell invasion, and invasion was higher in the FTY720 + S1P group than the Con + S1P group (Fig. 6B; P<0.01). These results suggested that S1PR2 inhibited migration of cancer. Following transfection with S1PR2-specific siRNA, SW480 cell proliferation was significantly increased (Fig. 6E; P<0.05 and P<0.001, Con vs. si-S1PR and Con + S1P vs. si-S1PR + S1P, respectively), while there was no significant difference in SW480 cell viability following S1P stimulation.

Discussion

CRC is associated with mortality and its 5-year survival rate is associated with clinical stage (1). The early discovery and diagnosis of aggressive carcinoma is key to improving survival rate. There is a need to identify novel molecular therapeutic targets for diagnosis and treatment of CRC. S1P is synthesized by SPHKs and binds S1PR1-5 to elicit downstream responses, thus regulating cellular processes, including proliferation, migration and metastasis (9,19,31-34). Previous studies have suggested that aberrant expression of S1P signalling is involved in inflammation, angiogenesis and lymphocyte trafficking (35-37). The synthesis and breakdown of S1P are dysregulated in multiple types of cancer, such as hepatocellular carcinoma and ovarian cancer (7,38,39). To the best of our knowledge, however, the underlying mechanisms are not fully understood. The present study aimed to investigate the role of SPHK/S1P/S1PR signalling in colon cancer.

SPHK1 and SPHK2 are key regulators of sphingolipid-mediated functions (19,35). Previous studies have shown that expression of SPHK1 is upregulated in breast cancer and is associated with drug resistance and poor prognosis (40-43). The present results suggested that SPHK1 expression in colorectal tissue was upregulated during malignant transformation of human CRC. This was consistent with previous reports that peripheral circulation and tumour S1P levels are increased in colon cancer (44,45). The present results demonstrated that synthesis of S1P was dysregulated in CRC, which lead to increased production of S1P. The bioactive lipid S1P serves a key role in cancer by promoting cell proliferation and invasion and angiogenesis (16,46-48). S1P levels in plasma from patients with CRC and healthy individuals were measured using ELISA to determine whether circulating S1P levels were a useful biomarker; levels of S1P were significantly elevated in patients with CRC patients. These results are consistent with a previous report that S1P is upregulated in patients with other types of cancer, liver or neural system tumours (49).

S1P exerts its signalling function in an autocrine or paracrine manner via S1PR1-5 (11). The results of the present study suggested that expression levels of S1PR2 and S1PR4 were significantly downregulated in CRC, while expression of S1PR1 was upregulated, which is consistent with previous studies on glioblastoma and hepatocellular carcinoma (24,30). Wound

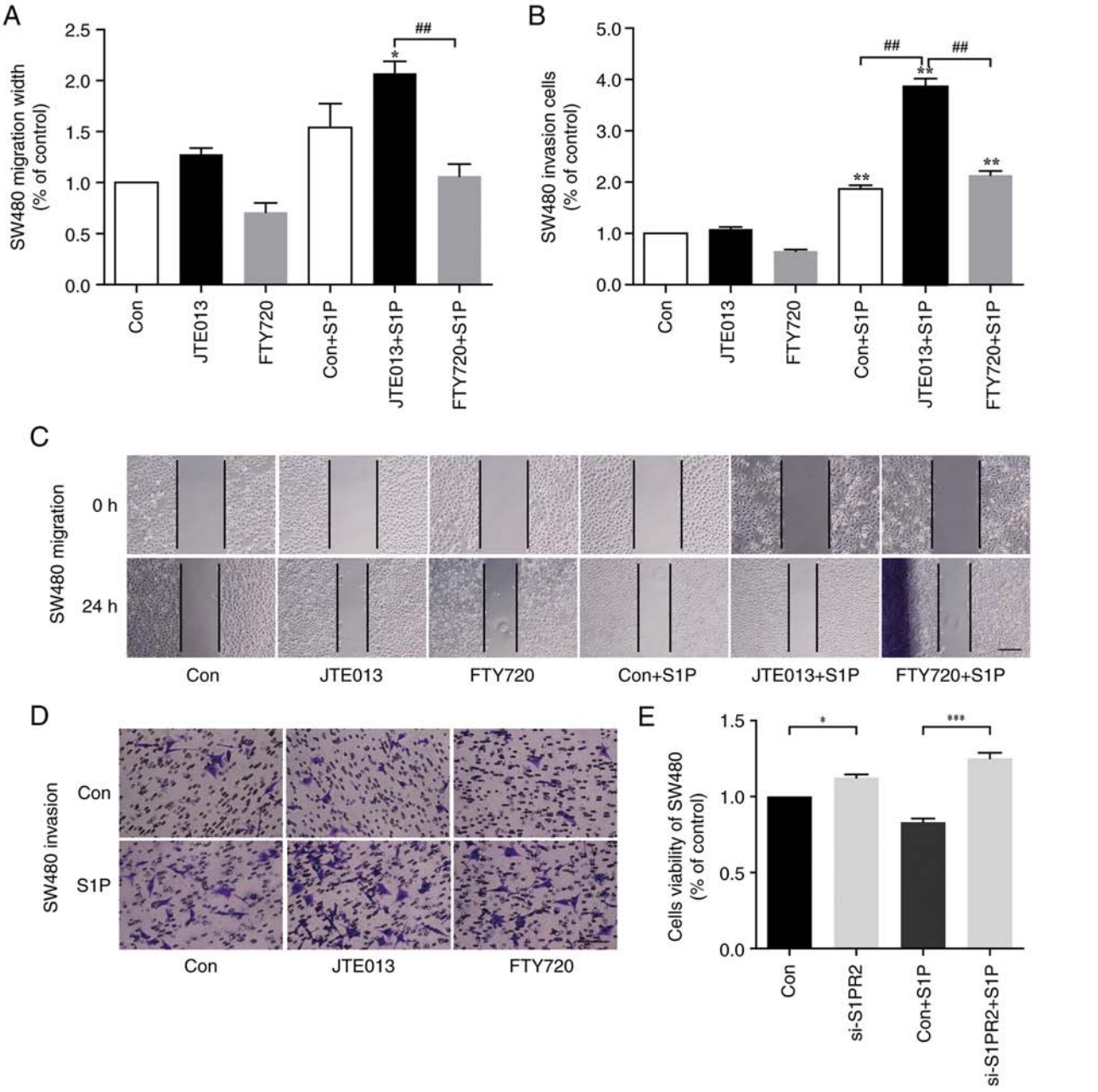

Figure 6. Role of S1PR2 in cell migration, invasion and proliferation. (A) Wound healing assay was performed in the presence or absence of 1 μM S1P, 5 μM JTE013 and/or 5 μM FTY720. *P<0.05, JTE013 vs. JTE013 + S1P; $^{\#\#}$P<0.01, JTE013 + S1P vs. FTY720 + S1P. (B) Transwell assay was performed in the presence or absence of 1 μM S1P, 5 JTE013 and/or 5 μM FTY720. Data are expressed as the percentage of invading cells relative to untreated control. **P<0.01, Con vs. Con + S1P, JTE013 vs. JTE013 + S1P, FTY720 vs. FTY720 + S1P. $^{\#\#}$P<0.01, JTE013 vs. JTE013 + S1P vs. FTY720 + S1P. (C) Representative images of the wound healing assay. Scale bar, 200 μm. (D) Representative images of the Transwell assay. Scale bar, 100 μm. (E) MTT assay was performed to detect human CRC cell viability. SW480 cells were transfected with S1PR2-specific siRNA and treated in the presence or absence of 1 μM S1P. n=3. *P<0.05, Con vs. si-S1PR2, ***P<0.001, Con +S1P vs. si-S1PR2 + S1P. S1PR, S1PR, sphingosine-1-phosphate receptor; si, small interfering; Con, control.

healing assay demonstrated that LOVO cells treated with exogenous S1P showed a significant increase in cell migration, whereas migration of SW480 cells was inhibited. The effect of S1P on the migration of different colon cancer cell lines may depend on receptor subtype distribution on the cell surface. S1P transduces signals by binding plasma membrane G-protein-coupled receptors. The expression of S1PR is tissue-specific and S1P signalling produces different cellular outcomes (11). The present study demonstrated that expression levels of S1PR subtypes were different in the two cell lines, which may contribute to the different results. mRNA expression levels of S1PR2 and S1PR3 were high in SW480 cells, whereas S1PR2 was expressed at low levels in LOVO cells. The effect of S1P on migration was based on the expression of S1PR2. This was consistent with the increased migration following inhibition of S1PR2. Expression of S1PR2 mRNA was increased in CRC tissue but decreased in colon cancer cells, suggesting that expression of different types of S1PR was associated with malignancy potential and confirming that SW480 has a low metastatic ability.

S1PR2 levels have previously been reported to be decreased in colon and prostate cancer compared with normal tissue and are associated with poor patient prognosis (15,50). To the best

of our knowledge, however, no correlation has previously been found between S1PR2 expression and disease-free survival in human CRC. Therefore, it was hypothesized that S1PR2 may also be involved in the progression and development of CRC. Here, expression of S1PR2 in 55 patients with CRC was detected at the protein and mRNA levels by western blotting, IHC and RT-qPCR. Expression of S1PR2 was downregulated in CRC, as evident in 32/55 cases in western blotting and 33/50 cases in IHC. The gene expression of S1PR2 was significantly downregulated in tumour tissue compared with normal surgical margin tissue.

S1PR2 serves a key role in cancer progression (16). Salas *et al* (34) suggested that systemic SPHK1/S1P-regulated metastatic potential via inhibition of S1PR2 signalling further elevated breast cancer metastasis suppressor 1 level and suppressed lung tumour metastasis. Here, S1PRs were expressed at different levels in colon cancer cell lines. Treatment of cells with S1PR2 antagonist JTE013 in the presence of S1P enhanced promotion of cell migration and invasion compared with the control group. Moreover, treatment with S1PR2 siRNA significantly enhanced cell proliferation compared with the control group. Therefore, the promoting effect of S1P was enhanced by inhibition of S1PR2 expression, which confirmed the hypothesis that loss of S1PR2 in colon cancer may contribute to cell migration and invasion.

FTY720 (also named Fingolimod) is an immunosuppressant agent used to treat multiple sclerosis and has shown anticancer activity (13,51) against glioblastoma and haematological malignancy (37). FTY720 decreases cell viability and survival and inhibits cancer progression via downregulation of key nutrient transport proteins, selectively starving cancer cells to death (52). FTY720 interferes with the SPHK1/S1P/S1PR1 axis and suppresses the NF-κB/IL-6/Stat3 amplification loop and colitis-associated cancer in mice (21). Here, treatment with 5 μM FTY720 inhibited promotion of cell migration and invasion induced by JTE013. FTY720, a sphingosine analogue, primarily acts via S1PR1, 3, 4 and 5 to cause receptor endocytosis, which leaves S1P binding to S1PR2 alone, thus enhancing the role of S1PR2 in the inhibition of cell migration and invasion. This suggests that FTY720 may be useful for treating CRC in humans.

The present study had certain limitations. The present preliminary clinical study primarily focused on the role of S1PR2 in the proliferation and migration of colon cancer cell lines; due to the limited sample size, expression levels of the other four S1PRs were only measured by RT-qPCR rather than using the same methods as for S1PR2. The association between S1PRs and S1P was not fully elucidated; further investigation is required to determine the role of the SPHK/S1P/S1PR signalling pathway in colon cancer. Although the present study revealed that secretion of SPHK1 was upregulated in CRC tissue and S1P levels in serum of patients with CRC were higher than those in healthy individuals, the potential correlation between them was not assessed. Further experiments are required to verify whether CRC cells secreted S1P. Cell migration was assessed at only one time point; more time points need to be analysed and migration velocity should be determined in future. Animal experiments were not performed to confirm the present findings. The expression of molecules was only assessed by western blotting, IHC and RT-qPCR. More studies are needed to confirm the results of the present study. Basic molecular research involving downstream signalling pathways is also required.

In summary, the present study indicated that expression levels of S1PRs, particularly S1P2, were associated with migration and invasion of CRC cells. The present study identified a novel mechanism in which S1P inhibited tumour cell migration and invasion via the S1PR2-dependent pathway, suggesting that S1PR2 may be a therapeutic target for treatment of colon cancer.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ conceptualized and supervised the study, collected and analysed data and edited the manuscript. JY designed the methodology, performed the experiments, collected, analysed and visualized data and wrote the manuscript. QW performed the experiments, collected and analysed data, edited the manuscript and supervised the study. LS and YC performed the experiments. XZ and JY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All specimens were obtained for a previous study and were used in the present study with verbal consent from the patients and healthy donors. The study was approved by the Institutional Ethics Committee of Nanjing Medical University (approval no. 2016-SR-217).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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