MsrB1 Promotes Proliferation and Invasion of Colorectal Cancer Cells via GSK-3β/β-catenin Signaling Axis

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Abstract
Methionine sulfoxide reductase B1 (MsrB1) can catalyze both free and protein-bound R-methionine sulfoxides (R-MetO) to methionine (Met). It has been reported that MsrB1 plays an important role in the development of HCC and human bone osteosarcoma. However, little is known about the functions of MsrB1 in human colorectal cancer (CRC). Herein, we detected MsrB1 expression level in CRC tissue and cell lines, and investigated the effect of MsrB1 knockdown on CRC phenotypes and possible mechanisms involved in. The results showed that MsrB1 was highly expressed in both CRC tissues and cell lines, and that cell proliferation, migration and invasion were significantly inhibited, but apoptosis was increased after MsrB1 knockdown in colorectal cancer HCT116 and RKO cell lines, compared to control siRNA group. In addition, E-cadherin protein level was increased, vimentin and Snail protein were greatly decreased after knockdown of MsrB1 in cells. Furthermore, pGSK-3β (Ser9) and β-catenin protein levels were reduced, the promoter activity of TCF/LEF construction was inhibited after MsrB1 knockdown in cells, suggesting that GSK-3β/β-catenin signaling axis was involved in the tumorigenesis of CRC. In conclusion, the oncogenic role and related mechanisms of MsrB1 in CRC discovered in our work determined the potential role of MsrB1 as a biomarker and may provide a new target for clinical therapy of CRC.

Keywords
methionine sulfoxide reductase B1, colorectal cancer cells, cell proliferation, epithelial-mesenchymal transition, GSK-3β/β-catenin signaling pathway

Introduction
Methionine sulfoxide reductase B1 (MsrB1), a member of the selenoprotein family, contains a selenocysteine (Sec) residue in its catalytic site, and can specifically catalyze both free and protein-bound R-methionine sulfoxides (R-MetO) to methionine (Met). The existence of Sec makes seleno-protein endowed with an antioxidative function2–5. Previous works have confirmed that MsrB1 has close relationship with oxidative damage-related disease or disorder, such as aging, neurodegenerative disease, and diabetes6–12. Thus, as Sec involved in the composition, and its function in oxidative damage-related disease, the antioxidative role of MsrB1 has been deeply rooted. Recent studies have showed that MsrB1 is involved in the development of human hepatocellular carcinoma (HCC)13 and human bone osteosarcoma14, suggesting that MsrB1 plays an important role in the development of some types of cancers.
Tumorigenesis is a complex process during which oxidative damage on DNA leads to gene mutation, directly participating in the formation of polygenic diseases. MsrB1, emphasized as an antioxidant, participates in oxidative damage repair in biological molecules including DNA\(^4\). On the other hand, Met is a molecule directly related with methyl supply in vivo\(^5\). As methyl is essential for DNA synthesis, and MsrB1 can catalyze MetO to Met, there comes the statement that MsrB1 impacts DNA synthesis/cell proliferation in cancer cells. In fact, a few studies have revealed that MsrB1 plays specific roles in the development of tumors\(^{13,14}\), indicating undiscovered features of MsrB1 in tumorigenesis. Colorectal cancer (CRC) is one of the most common cancers worldwide and causes half a million deaths each year. It has been reported to be associated with multiple environmental and lifestyle factors\(^6\). However, little is known about the role of MsrB1 in CRC development.

In this study, we detected MsrB1 expression level in CRC tissue and cell lines, and investigated the effect of MsrB1 knockdown on CRC phenotypes. We found that MsrB1 was highly expressed in CRC tissues and cell lines, and that cell proliferation, migration and invasion were significantly inhibited, but apoptosis was increased at meantime after MsrB1 knockdown in colorectal cancer HCT116 and RKO cell lines, compared to control siRNA group. Based on these alterations on phenotypes, crucial molecules involved in classic tumor signaling pathway was investigated, trying to explore the mechanism behind the phenotype alterations. Conclusively, MsrB1 could promote cell proliferation, cell migration and invasion of CRC cells as an oncogene, and GSK-3\(\beta\)/\(\beta\)-catenin signaling pathway was involved in the process. The oncogene role and related mechanisms of MsrB1 in CRC discovered in our work determined the potential role of MsrB1 as a biomarker and may provide a new target for clinical therapy of CRC.

**Materials and Methods**

**Small Interfering RNA (siRNA)**

Small interfering RNAs (siRNAs) for MsrB1 were purchased from Gene Pharma Inc. (Shanghai, China). A set of two siRNAs with different sequences for MsrB1 were used to knock down MsrB1 expression in cells. For MsrB1, the sequence pairs were 5'-GGAGCACAATAGATCTGGAATT-3' and 5'-UUCAGAUCUUUGUGCUCCCTT-3' (siMsrB1-1); 5'-GGGCGCCAGGACAAUGATTT-3' and 5'-UCUAUUUGGUCCCGACGCTTT-3' (siMsrB1-2)\(^12\).

**Plasmids**

Wild type MsrB1 (Sec) (Catalog Number: EX-U1161-M35), mutant type MsrB1 (Cys) (Catalog Number: CS-U1161-M35) and empty vector (Catalog number: EX-NEG-M35) plasmids were purchased from GeneCopoeia Inc, (Rockville, MD, USA).

**Cell Culture and Transfection**

Human CRC cell lines HCT116 and RKO were maintained in DMEM (Hyclone, Logan, UT, USA) with 10% FBS (PAN-Biotech, Adenbach, Germany) and penicillin-streptomycin at 37°C, 5% CO\(_2\). When cells grew to ~60% confluence, transfection was performed in 6-cm tissue culture dishes or 24-well plates (BD company, Franklin Lakes, NJ, USA) using Lipofectamine RNAiMax or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturer’s protocol.

**RNA Isolation and Real-time PCR**

Total RNA was isolated from cultured cell using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturer’s instructions. RNA (1 \(\mu\)g) was reverse transcribed using SuperScript reverse transcriptase (Bio-Rad, Hercules, CA, USA) to generate first-strand cDNA. Real-time PCR experiments were performed using a DNA Engine Opticon 2 (MJ Research, Watertown, MA, USA). SYBR Green PCR Master Mix kit (Toyobo, Japan) was used for PCRs. Primers used are listed as follows: human MsrB1, sense, 5'-GACGTACACCCCTCAGCTT-3', and antisense, 5'-AGCTACTCTGGACACAGATT-3'; human GAPDH, sense, 5'-CATGGCCAGCTGCTCCCGTGA-3'; and antisense, 5'-CTGCAAATGGCACCCCTGGT-GAC-3'\(^17\). The relative expression levels were calculated using 2\(^{-\Delta\Delta CT}\) rules.

**Protein Extract and Western Blot Analysis**

Protein extracts from CRC cells were prepared in radio immunoprecipitation assay lysis buffer (sc24948; Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplied with PMSF, protease inhibitor mixture, sodium orthovanadate, or RNase inhibitor. Lysates proteins were quantified using a Bio-Rad protein assay kit. 40 \(\mu\)g of proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes and blocked with 5% nonfat milk in TBS-T (20 mMTris, 500 mMNaCl, and 0.1% Tween 20) at room temperature for 1 h with rocking. The membranes were probed with primary antibodies overnight at 4°C. After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies (sc-51602; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% nonfat milk/TBS-T at room temperature for 1 h. The protein–Ab complex was detected by Western blotting luminol reagent (ECL substrate number 32106; Thermo Fisher Scientific, Rochester, NY, USA). All primary antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were listed as follows: anti-MsrB1 (sc-135558), anti-E-cadherin (sc-8426), anit-vimentin (sc-6260), anti-Snail/SNAI1 (sc-271977), anti-GSK-3\(\beta\) (sc-81462), anti-phospho-GSK-3\(\beta\) (sc-81495), anti-\(\beta\)-catenin (sc-7963), anti-GAPDH (sc-47724).
Cell Proliferation Assay

Cells were cultured in 96-well plates (15,000 cells/0.2 ml per well). After siRNA transfection, 5 μl (along with 95 μl DMEM) reagent of Cell Counting Kit-8 (CCK-8) (Bimake, China) was added to each well. After incubating for 1 h, absorbance values were determined spectrophotometrically at 450 nm on a Microplate Reader (BIO-TEK, Rockville, MA, USA).

Colony Formation Assay

As previously described. Briefly, cells were cultured in 96-well plates (1,000 cells/0.2 ml medium per well). Considering the temporary nature of the siRNA transfection, new transfection was processed for every 72 h. Colony formation were then observed after 10 days. Specifically, cells were washed with PBS for 3 times, fixed with 75% ethanol for 15 min, stained with 0.1% crystal violet for 15 min. Cells were washed and observed under an optical microscope (Leica, Wetzlar, Germany).

Flow Cytometry Analysis

For apoptosis detection, 1 × 10⁶ cells were collected, washed twice in ice-cold PBS. An Annexin-V-FITC apoptosis detection kit (KeyGEN Biotech, Nanjing, China) was used for the quantitative analysis of cell apoptosis. Cells are considered viable when both FITC Annexin V and PI were negative; when in early apoptosis, FITC Annexin V positive and PI negative; in late apoptosis or already dead, both FITC Annexin V and PI positive. The percentage of apoptotic cells was analyzed in a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

For cell cycle detection, 1 × 10⁶ cells were collected, washed twice in ice-cold PBS and fixed in 70% ice-cold ethanol overnight at 4°C, then cells were washed twice in ice-cold PBS and digested with RNase A (10 μg/ml) at 37°C for 30 min. Cells were stained with 10 μg/ml of propidium iodide (Sigma-Aldrich, St Louis, USA) for 3 min at room temperature before testing. DNA contents of cells (10,000 cells per experimental group) were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

5-Ethynyl-2’-Deoxyuridine (EdU) Assay

Cell proliferation was also detected by an EdU Cell Proliferation Assay Kit (RiboBio, Guangzhou, China) according to the manufacturer’s instructions. The proportion of cells that incorporated EdU was determined with a fluorescence microscope (Leica, Wetzlar, Germany).

Wound-Healing Assay

Monolayer cells in 6-well plates (2.0 × 10⁵ cells/well) were scratched with a 200 μl pipette tip after the siRNA transfection, and the medium was replaced with a serum-free medium. Each scratch wound was recorded with a microscope (Leica, Wetzlar, Germany) at the same position at 0 h and 24 h, respectively. The experiments were conducted in triplicate.

Transwell Assay

The capacity of cell invasion was assessed by transwell assay. First, the transwell chamber was covered with 40 μl Matrigel (BD company, Franklin Lakes, NJ, USA). The colon cancer cells (0.5 × 10⁵ cells/chamber) were seeded on the upper chamber and incubated with serum-free culture medium. 800 μl medium was then added in the lower chamber with 30% serum. After a 48-h culture, the colon cancer cells in the upper chamber were washed twice. The lower cells were fixed with 4% paraformaldehyde (Aladdin, China) for 25 min and stained with 0.4% crystal violet stain (Amresco, Solon, OH, USA) for 5 min. Five randomly microscopic fields were selected for analysis with a microscope (Leica, Wetzlar, Germany).

Immunohistochemistry (IHC)

CRC tissue microarray slide (BC05118c) was purchased from Alenabio Bioscience Corporation (Xi’an, China), containing adenocarcinoma (80 cases, including T3N0M0 5/T3N1M0 2/T3N0M0 15/T3N1M0 14/T3N2M0 1/T4N0M0 8/T4N1M0 3/T4N2M0 3), and normal adjacent colon tissue (NAT) (20 cases). After deparaffinization, tissue sections underwent antigen retrieval by autoclaving the slides for 3 min in 10mM citrate buffer (pH6.0). Sections were pretreated with 0.3% H2O2 in methanol for inactivation of endogenous peroxidase, and nonspecific interactions were blocked for 30 min using a 5% goat serum solution. The primary antibody was diluted with 5% goat serum solution, applied to the slides and incubated overnight at 4°C. The slides were incubated subsequently with a biotinylated secondary antibody, and antigen-antibody complexes were detected with an avidin-biotin complex detection kit (Solarbio, Beijing, China), and developed with diaminobenzidine. The sections were counterstained with hematoxylin. The antibody used for staining was anti-MsrB1 antibody (sc-135558; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunofluorescence assay

As described previously. The anti-β-catenin antibody (sc-7963, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. The cells were incubated with FITC-conjugated secondary antibody (ThermoFisher, Rochester, NY, USA). The nucleus was stained by DAPI (ThermoFisher, Rochester, NY, USA).
Dual Luciferase Reporter Assay

The TCF/LEF reporter plasmid (TOPFlash) was originally purchased from Addgene (Cat #12456; Cambridge, MA, USA). The Renilla luciferase plasmid was obtained from Promega (Madison, WI, USA). Cells transfected with control siRNA or MsrB1siRNA were plated in 24-well plate and transfected with 200 ng of TOPFlash along with 20 ng of pRL-TK to normalize the transfection efficiencies. At 48 h after transfection, cells were collected, and luciferase activities were measured by the dual-luciferase reporter assay kit (Promega, Madison, WI). The luciferase activities are represented as normalized TOPFlash/pRL value. The results are expressed as the mean ± SD of three replicates.

Statistical Analysis

All results are expressed as the mean ± standard deviation (SD). The amount of changes in all experiments are reported as fold changes or percentages. The statistical significance of difference between groups was evaluated by analysis of variance, followed by Student’s t-test. Significance was designated as follows: *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Results

Alteration of MsrB1 Expression in CRC Patient Samples and Cell Lines

MsrB1 expression level in CRC tissue samples was detected by IHC, using CRC tissue microarray slide. As shown in Fig. 1A, MsrB1 expression level was higher in tumor tissue than that in the normal adjacent colon tissue. Different CRC cell lines were also used to determine MsrB1 expression alteration by Western blot. The result indicated that MsrB1 expressions were upregulated in all the CRC cell lines, compared to that in the human intestinal epithelial cells (HIECs) (Fig. 1B, C).

Figure 1. MsrB1 protein expression levels in colon cancer samples. (A) MsrB1 protein level detected by IHC assay in colon cancer tissues and corresponding non-cancerous colon tissues. Representative IHC images were shown. N: noncancerous colon tissue; T: colon tumor tissue. Magnification: X100. (B) MsrB1 protein levels by Western blot in colorectal cancer cell lines. (C) The relative MsrB1 protein levels in cells in (B) were quantified. GAPDH was used as a loading control. The ratio of MsrB1/GAPDH protein in human intestinal epithelia cells (HIEC) was normalized to “1.” Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, compared to HIEC.

MsrB1 Knockdown on Cell Proliferation

We firstly designed series of siRNAs for MsrB1 knockdown in HCT116 and RKO colon cancer cell lines, and the knockdown efficiency was remarkable compared with siCtrl group (Fig. 2A–C). The viability of colorectal cancer cell HCT116 and RKO by CCK-8 assay was significantly decreased after MsrB1 knockdown (Fig. 2D, E), compared to siCtrl control group. To further test the effect of MsrB1 on cell proliferation, the colony formation assay and Edu incorporation assay were performed, as shown in Fig. 2, colony number (Fig. 2F–H) and Edu positive cells (Fig. 2–K) were remarkably reduced after knockdown of MsrB1 in cells, respectively, compared to control siCtrl group. Together, these results suggest that knockdown of MsrB1 could inhibit cell proliferation of CRC cells.

MsrB1 Knockdown Leads to Cell Apoptosis

To determine whether the inhibition of cell proliferation caused by MsrB1 knockdown is due to cell cycle arrest or apoptosis, flow cytometry was used to detect cell cycle profile and apoptosis. As shown in Fig. 3A, B, no alterations in percentages of G1, S and G2/M phase of cell cycle profile were observed in HCT116 and RKO cancer cell lines after MsrB1 knockdown; while annexin V and propidium iodine–positive cells were increased from about 4.5% to 20% in HCT116 cells, from 4.9% to 17% in RKO cells (Fig. 3C, D) after knockdown of MsrB1, compared to corresponding siCtrl control groups, respectively, indicating that the inhibition of proliferation by MsrB1 knockdown in cells is mainly due to apoptosis, but not cell cycle arrest.

MsrB1 Knockdown Inhibits Cell Migration and Invasion in Vitro

We further explored the effect of MsrB1 on colon cancer cell migration and invasion. Wound-healing assay showed that knockdown of MsrB1 impeded the migration in monolayer cultured HCT116 and RKO cells (Fig. 4A–C). In addition,
by transwell assay, we observed the number of invaded cells was obviously attenuated in the siMsrB1 groups compared to siCtrl group (Fig. 4D, E).

**MsrB1 Knockdown Inhibits EMT and GSK-3β/β-Catenin Signaling Pathway in CRC**

Epithelial-mesenchymal transition (EMT) plays an important role in tumor invasion and migration. To investigate the mechanisms by which knockdown of MsrB1 inhibits cell migration and invasion, we analyzed the effects of MsrB1 knockdown on EMT by detecting EMT-related factor expression by western blot in HCT116 and RKO cells. As shown in Fig. 5A, B, MsrB1 knockdown increased E-cadherin expression level, and decreased Vimentin and Snail expression levels in both cell lines, compared to siCtrl control groups, suggesting that MsrB1 knockdown inhibits EMT in CRC cells. It has been showed that deregulation of
Wnt signaling pathway is a major contributor to colorectal carcinogenesis. The Wnt/β-catenin is activated in most of CRCs (about 90%), leading to the β-catenin accumulation in the nucleus20. We next detected β-catenin expression level after knockdown of MsrB1 in HCT116 and RKO cells. As expected, β-catenin protein levels were significantly decreased (Fig. 5C, D) in cell lines after knockdown of MsrB1, compared to siCtrl control groups. pGSK-3β (Ser9, an inactive form), an upstream protein of β-catenin, was also decreased in two cell lines after knockdown of MsrB1. In addition, β-catenin immunostaining results showed that the translocation of β-catenin protein was decreased from cytoplasm to nucleus after knockdown of MsrB1 in the cells (Fig. 6A, B). Further, knockdown of MsrB1 inhibited the promoter activity of TCF/LEF construction (Fig. 6C), compared to siCtrl group, indicating that knockdown of MsrB1 could inhibit CRC via GSK-3β/β-catenin signaling pathway.

The Selenocysteine (Sec) Residue in the Catalytic Center of MsrB1 Plays an Important Role in Cell Proliferation, Migration and Invasion

To determine whether the selenocysteine (Sec) residue in the catalytic center of MsrB1 was important for cell proliferation, migration and invasion of CRC cells, we transfected cells with plasmid expressing wild type MsrB1 protein (Sec), and plasmid expressing mutant type MsrB1 protein (Cys), in which the selenocysteine (Sec) residue in the catalytic center of MsrB1 was mutated to cystein (Cys) residue, respectively, then performed CCK-8, colony formation, wound healing and transwell assays. The results showed that the extent of cell proliferation, migration and invasion was more increased in wild type MsrB1 group than that in mutant type MsrB1 group (Fig. 7A–E), suggesting that the selenocysteine (Sec) residue in the catalytic center of MsrB1 plays a crucial role in these processes.
Figure 4. Effects of MsrB1 knockdown in colorectal cancer cell lines on migration and invasion. (A–C) Wound healing assay was analyzed in HCT116 and RKO cells. The percentage of wound closure in three independent experiments was showed in histograms. (D, E) Transwell assay was performed to observe cell invasion of HCT116 and RKO cells, as described in the materials and methods. The invaded cells of three independent experiments were summarized in histograms. **P < 0.01, ***P < 0.001.

Figure 5. Western blotting for proteins involved in EMT and GSK3β/β-catenin signaling pathway in colorectal cancer cells after knockdown of MsrB1. (A, B) Western blotting for E-cadherin, vimentin and Snail proteins. (C, D) Western blotting for GSK3β, phosphorous GSK3β and β-catenin proteins. GAPDH was used as a loading control. The ratio of target protein/GAPDH protein in siCtrl group was normalized to “1.” **P < 0.01, ***P < 0.001.
an important role in cell proliferation, migration and invasion of CRC.

**Discussion**

MsrB1, firstly reported as a member of the Msrs family, can catalyze R-MetO to Met. Detection on the MsrB1 composition determined that a Sec locates in the catalytic site, making it a selenoprotein, endowed with an antioxidative ability. Thus, MsrB1 has been reported involving in the pathological process of oxidative damage-related disease, such as aging, neurodegenerative diseases, and diabetes. Considering the catalytic property that MsrB1 can reverse MetO to Met, together with the unique role that Met directly supplies methyl, as one of the components of DNA in vivo, we could infer that MsrB1 involves in the process of DNA synthesis and cell proliferation. Recently, several studies have revealed that MsrB1 plays specific roles in different types of cancers, indicating feature of MsrB1 in tumorogenesis. However, the specific role of MsrB1 in the occurrence and development of CRC is not very clear.

**Figure 6.** Knockdown of MsrB1 inhibits GSK-3/β-catenin signaling in colorectal cancer cell lines. (A, B) Knockdown of MsrB1 inhibited nuclear translocation of β-catenin. Immunofluorescence staining of β-catenin in HCT116 and RKO cells after knockdown of MsrB1 with siRNAs. (C) The relative LEF/TCF promoter activities in HCT116 and RKO cells after knockdown of MsrB1 with siRNAs. *P < 0.05, **P < 0.01, compared to siCtrl control group.

**Figure 7.** The selenocysteine (Sec) residue in the catalytic center of MsrB1 plays an important role in cell proliferation, migration and invasion of CRC cells. HCT116 and RKO cells were transfected with empty vector (EV), vector expressing wild type MsrB1 protein (Sec), vector expressing mutant type MsrB1 protein (Cys), in which the selenocysteine (Sec) residue in the catalytic center of MsrB1 was mutated to cystein (Cys) residue, respectively, then CCK-8 (A), colony formation (B and C), wound healing (D) and transwell (E) assays were performed. Data are expressed as mean ± SD of at least 3 independent experiments. * P < 0.05, ** P < 0.01, ***P < 0.001.
In our present study, we found that MsrB1 may work as an oncogene in colon tumor progression. Knockdown of MsrB1 inhibits the proliferation, migration and invasion, and promotes apoptosis of colorectal cancer cell lines, mitigates EMT process. In addition, GSK-3b/β-catenin signaling pathway may be involved in CRC development associated with MsrB1. Together, MsrB1 could promote the proliferation, migration and invasion of CRC cells as an oncogene, and GSK-3b/β-catenin signaling pathway was involved in the process. Our results determine the potential role of MsrB1 as a biomarker and may provide a new target for clinical therapy of CRC.

Previously, the functional study of MsrB1 mainly focused on Drosophila and human lens epithelial cells. More recently, some researchers have begun to study the function of MsrB1 in cancer biology. In the present study, MsrB1 protein expression levels were significantly increased in both CRC tissues and cell lines (Fig. 1). Among CRC cell lines in Fig. 1, the expression level of MsrB1 of HCT116 cell was the most highest, while the level of MsrB1 of Lovo cell was the lowest, compared to HIEC cell line. So, in this study, we chose HCT 116 and RKO cells to further investigate the role of MsrB1 in colorectal cancer cells. Our findings are consistent with the study showing that MsrB1 expression is upregulated in HCC tissues compared with paratumor tissues, indicating that MsrB1 may contribute to development of CRC.

One of the hallmarks of cancer cells is uncontrolled or increased cell proliferation. It has been reported that cell cycle regulators play an important role during cell proliferation. In our study, knockdown of MsrB1 in HCT116 and RKO cells inhibited cell viability, colony formation and Edu incorporation, compared to control siRNA cells group (Fig. 2), suggesting that MsrB1 could promote proliferation of CRC cells. Interestingly, at mean time, no cell cycle changes were observed by flow cytometry (Fig. 3A, B), while apoptotic rate was significantly increased after knockdown of MsrB1 in HCT116 and RKO cells (Fig. 3C, D), indicating that MsrB1 promotes cell proliferation probably due to inhibition of apoptosis without affecting cell cycle. Previous work demonstrated that knockdown of MsrB1 inhibits cell proliferation and induces apoptosis in HCC cells, which are similar to our findings. However, G1 phase of cell cycle was arrested in that study. The difference of distribution of cell cycle phase after knockdown of MsrB1 in cells between our experiment and previous study is likely due to different types of cancer cell lines used, which needs to be further investigated.

Invasion and metastasis are another important biological characteristics of malignant tumors, which are closely related to stages in the growth and development of malignant tumors. Totally, it was considered that invasion is the prelude to metastasis, and metastasis is the result of invasion. Here, we demonstrated that knockdown of MsrB1 inhibits migration and invasion of CRC cells (Fig. 4), which is also consistent with previous study, indicating an oncogene characteristic of MsrB1.

Based on the phenotypic changes stated above, molecular mechanisms behind were investigated in our research. Generally, several cell signal pathways were detected, indicating that Wnt/β-catenin pathway was closely linked with the phenotypic changes. It has been confirmed that in approximately 90% of CRCs Wnt/β-catenin pathway is activated, and phosphorylation of serine 9 in GSK-3b results in β-catenin accumulation in nucleus, affecting cell phenotypes by regulating downstream genes expression. In addition, β-catenin can be regulated by E-cadherin, a protein related with both cell proliferation and migration/invasion. It is worth mentioning that E-cadherin is of importance on maintaining multicellular structure, which is related with the migration/invasion ability of CRC cells. An upregulation of E-cadherin was shown after MsrB1 knockdown. Meanwhile, a downregulation in vimentin expression was observed, a molecule confirmed to destroy cell polarity, weaken adhesion, spindle cell shape, and enhance cell motility. Besides, downregulation on Snail, an EMT-related transcriptional factor (EMT-TF), which inhibits the expression of E-cadherin, was also observed in CRC cells after MsrB1 knockdown (Fig. 5A, B). All the molecular alterations point to a transformation of CRC cells from invasive to a benign state, indicating an oncogenic effect of MsrB1 in CRC (Fig. 5). Our finding is consistent to previous work showing that knockdown of MsrB1 in HCC cell lines inhibits the acquisition of migration/invasion ability.

Last but not the least, it is well-documented that MsrB1 exhibits the highest methionine-R-sulfoxide reductase activity among MsrB family (MsrB1, MsrB2, MsrB3a, and MsrB3b) because of the existence of Sec in its catalytic site. Combined with our present work, whether the Sec contained in MsrB1 has direct relationship with CRC tumorigenesis or development is not known. Mutated MsrB1 (Sec to Cys) was overexpressed by transfecting plasmid in CRC cells, the extent of cell growth, colony formation, migration and invasion was significantly lower than that in wild type MsrB1 group (Fig. 7). The result suggests that Sec in catalytic site of MsrB1 is tightly associated with the progression of CRC. The related molecular mechanism remains to be further investigated.

In summary, in our present work, we determined that MsrB1 plays its specific role in the development of CRC via GSK3β/β-catenin pathway. Thus, our findings provide insight into the MsrB1 functions in CRC and clinical treatment strategy for developing MsrB1 as a potential biomarker and clinical therapeutic target for CRC. However, in vivo study about the MsrB1 functions, and the specific mechanisms by which MsrB1 interacts with components of GSK-3β/β-catenin signaling pathway to promote the CRC need to be further studied.

Author Contribution

Xiao-Yu Chen, and Sheng-Yong Yang are authors contributed equally to this work.
Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval
This study was approved by Ethics Committee of Chongqing Medical University, Chongqing, China.

Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects (Note: CRC tissue microarray slide in this study was directly purchased from AlenabioBioscience Corporation as described in materials and methods section).

Statement of Informed Consent
We confirm that there are no human subjects in this article and informed consent is not applicable (Note: CRC tissue microarray slide in this study was directly purchased from AlenabioBioscience Corporation as described in materials and methods section).

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