miR-885-5p upregulation promotes colorectal cancer cell proliferation and migration by targeting suppressor of cytokine signaling

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Abstract. The aim of the present study was to investigate the role of microRNA (miR)-885-5p in colorectal cancer cell proliferation and migration, and to determine the possible underlying molecular mechanisms. The expression of miR-885-5p in colorectal cancer tissue and cells was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression levels of three suppressor of cytokine signaling (SOCS) factors were detected by RT-qPCR and western blotting. The effects of miR-885-5p on tumor cell proliferation and migration were studied using MTT and Transwell assays, respectively. Additionally, the expression levels of epithelial-mesenchymal transition (EMT)-related proteins (N-cadherin, E-cadherin, vimentin and Snail) were detected by RT-qPCR and western blot analysis. Furthermore, the target of miR-885-5p was predicted and confirmed using a luciferase reporter assay. miR-885-5p was demonstrated to be upregulated and SOCS was downregulated in colorectal cancer tissue, and cells. miR‑885‑5p suppression significantly inhibited tumor cell proliferation and migration, promoted E-cadherin expression, and inhibited the expression levels of N-cadherin, vimentin and Snail. Further studies showed that SOCS5, SOCS6 and SOCS7 were direct targets of miR-885-5p. The results suggest that miR-885-5p suppression inhibited cell proliferation and migration, and the EMT process by targeting SOCS5, SOCS6 and SOCS7 genes in colorectal cancer. miR-885-5p and SOCS may be used for the diagnosis and treatment of colorectal cancer.

Introduction

Colorectal cancer is the third most common type of malignancy, besides it is the fourth leading cause of cancer-associated mortality worldwide (1). Distant metastasis is the major cause of morbidity and mortality in patients with cancer, especially in colorectal cancer (2). In colorectal cancer ~90% of mortalities are caused by metastatic dissemination (3). Therefore, it is of great importance to understand the key underlying molecular mechanisms implicated in the metastatic process of colorectal cancer and to identify novel biomarkers that may aid in predicting prognosis, treatment outcomes, and the metastasizing propensity of the tumor.

Numerous studies have investigated the role of various genes in metastasis (4). MicroRNAs (miRNAs) are small noncoding RNAs that play critical roles in regulating gene expression (5). They are able to function as tumor suppressors or oncogenes, and are involved in multiple biological processes, including cell proliferation, differentiation, apoptosis, as well as metastasis (6). miRNAs have recently been identified to be potential biomarkers for numerous types of cancer, including colorectal cancer (7,8). For instance, miR-200c, miR-21 and miR-34b have been demonstrated to play pivotal roles in regulating metastatic behavior in colorectal cancer (9-11). A recent study demonstrated that miR-885-5p levels are upregulated in the serum of patients with colorectal cancer liver metastasis, suggesting that it may be a potential colorectal cancer metastasis-specific miRNA biomarker (12). However, the mechanism underlying the effect of miR-885-5p dysregulation on colorectal cancer metastasis remains unclear.

In the present study, the expression of miR-885-5p in colorectal cancer tissues and cells, as well as the effects of miR-885-5p on colorectal cancer cell proliferation, and migration were investigated. Additionally, the aberrant expression of suppressor of cytokine signaling (SOCS), an important negative regulator of cytokine and growth factor signaling, has been reported to critically affect cancer metastasis (13). Therefore, the expression levels of three SOCS factors were also investigated in colorectal cancer. Furthermore, the expression levels of target genes of miR-885-5p were investigated in order to determine the underlying mechanism of miR-885-5p in colorectal cancer metastasis.
Patients and methods

Patients. A total of 16 patients (9 males and 7 females, average age 57.3±6.4 years old) who were diagnosed with colorectal cancer between February 2013 and January 2016 at Liaoning Cancer Hospital and Institute, Cancer Hospital of China Medical University (Shenyang, China) were enrolled in the present study. The diagnosis of colorectal cancer was pathologically defined according to World Health Organization classification (14). The pathological tissues and matched adjacent normal colorectal tissues were separated from the same patient during colonoscopy, rapidly stabilized in RNA later reagent (Qiagen GmbH, Hilden, Germany) and then stored at -80°C. Patients were eligible if aged ≥18 years and ≤80 years and did not receive pre-operative chemotherapy and/or radiotherapy elective colorectal surgery for suspected carcinoma with primary anastomosis. Patients with excluded if they had: i) Distant metastasis; ii) synchronous tumors or multiple adenocarcinomas; and iii) received preoperative chemotherapy and/or radiotherapy. Written informed consent was provided from all patients and the study was approved by the Liaoning Cancer Hospital and Institute Protection of Human Ethics Committee.

Cell culture and cell transfection. The human colorectal cancer cell line SW480 (ATCC® CCL-228™) was purchased from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin, and 100 U streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The normal colon epithelial cell NCM460 was obtained through a Materials Transfer Agreement with INCELL Corporation LLC (San Antonio, TX, USA), which was cultured in Dulbecco’s modified Eagle medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% FBS. All cells were cultured in an atmosphere of 5% CO₂ at 37°C.

For cell transfection, miR-885-5p mimic (50 nM), inhibitor (150 nM) and scramble vectors, and the small interfering RNA (si)-SOCS5 (4 µg), si-SOCS6 (4 µg) and si-SOCS7 (4 µg) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Cell transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The primer sequences were as following: miR-885-5p mimic forward, 5'-UCCAUUAUCACUA CCCUGCCCU-3' and reverse, 5'-AGGCAGGGUAGUGUA AUGGAUU-3'; miR-885-5p inhibitor 5'-AGAGGCAGGGGUA GUGUAUCUU-3'; si-SOCS5 forward, 5'-GCUGUACUU UAUCGAGUAA-3' and reverse, 5'-UAUCUGUACUAG UAAAGCAGC-3'; si-SOCS6 forward, 5'-GGAGAAGUACUGU AAUAUAUA-3' and reverse, 5'-UAUCUGUACUAG UAAAGCAGC-3'; si-SOCS7 forward, 5'-AGAGGUGUUAAG UAGUAAGA-3' and reverse, 5'-UAUCUGUACUAG UAAAGCAGC-3'.

Cell proliferation assay. Cell proliferation ability was determined using an MTT assay. Briefly, cells transfected with silenced vectors at the logarithmic stage were cultured in DMEM/F12 supplemented with 10% FBS. Then, cells were seeded into the 96-well plates at a density of 5x10³ cells/well. After 24 h, cells were centrifuged at 6,000 x g for 5 min at 4°C and the supernatant was removed. Next, 20 µl MTT was added to the cell and they were cultured for an additional 4 h. Finally, 150 µl DMSO was mixed with the cells for 10 min. Absorbance of cells in wells was observed at 570 nm under an absorption spectrophotometer.

Clonogenic assay. After completion of siRNA transfection, cells were plated into the 60 mm tissue culture dishes at a cell density of 1x10⁵ cells/dish. Cells were grown in RPMI-1640 with 10% FBS for 14 days. After that, cells were fixed and stained with Diff-Quick, followed with air dry. Colonies were counted under microscope (IX83; Olympus Corporation, Tokyo Japan), and the cell number in each colony was at least 30 cells.

Cell migration assay. Transwell chambers were used for the cell migration assay. Cells in each group cultured for 48 h were incubated in serum-free RPMI-1640 medium containing 0.01% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h. Serum-free RPMI-1640 medium was added to the upper layer of the Transwell chamber and then air-dried at 4°C. After removing the medium from the cell cultures, 50 µl fresh serum-free medium containing 10 g/l BSA was added and cultured for 30 min at 37°C. Then, the cells were put into the 24-well plates and cultured with RPMI-1640 medium mixed with 10% FBS. After that, cells in Transwell were suspended with serum-free RPMI-1640 medium. After 48 h, cells were washed with PBS buffer to remove the upper cells on the microporous membrane and fixed in ice-cold 75% methanol at 4°C for 10 min. Finally, cells were stained with 0.1% crystal violet for 30 min at room temperature, and then decolorated with 33% acetic acid at room temperature for 15 min.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the isolated RNA was treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA). The concentration and purity of isolated RNA were measured with a spectrophotometer. Purified RNA (0.5 µg/µl) with nuclease-free water was used for cDNA synthesis using the PrimerScript 1st Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Expression levels of target genes were detected in an Eppendorf Mastercycler (Brinkmann Instruments, Inc.; Thermo Fisher Scientific, Inc.) using the SYBR ExScript RT-qPCR kit (Takara, China). Melting curve of amplification products was analyzed at the end of each PCR to confirm that only one product was amplified and detected. PCR was conducted under the following parameters: 1 predenaturation cycle of 1 min at 94°C, 34 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 2 min and a final extension at 72°C for 5 min. GAPDH was chosen as the internal control. The expression levels were calculated using the 2^−ΔΔCq method (15). Primers used for targets amplification are listed in Table I.

Western blotting. Cells were lysed using radioimmuno-precipitation assay buffer (Sangon Biotech Co., Ltd.)
containing phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and then centrifuged at 6,000 x g for 10 min at 4°C. The supernatants were collected and the total protein concentrations were measured using a bicinchoninic acid assay. Protein samples (20 µg) were separated on a 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes. Then, protein bands were blocked in PBST (0.1% triton in PBS) and probed with following primary antibodies: For SOCS5 (dilution, 1:1,000; cat. no. ab97283), SOCS6 (dilution, 1:1,000; cat. no. ab197335), SOCS7 (dilution, 1:1,000; cat. no. ab224589), E-cadherin (dilution, 1:1,000; cat. no. ab76055), N-cadherin (dilution, 1:1,000; cat. no. ab197335), Snail (dilution, 1:1,000; cat. no. ab8245), and vimentin (dilution, 1:1,000; cat. no. ab82846), and GAPDH (dilution, 1:5,000; cat. no. ab82846), and β-actin (dilution, 1:5,000; cat. no. ab97283), SOCS6 (dilution, 1:1,000; cat. no. ab18203), Snail (dilution, 1:1,000; cat. no. ab8245) at 4°C overnight. After that the membranes were incubated with the peroxidase-conjugated goat anti-rabbit IgG antibody (cat. no. ab6785; dilution 1:5,000) or peroxidase-conjugated goat anti-mouse IgG antibody (cat. no. ab6785; dilution 1:5,000; all Abcam, Cambridge, UK) for 1 h at room temperature. The immunoreactive protein bands were developed using enhanced chemiluminescence (ECL) substrates (Thermo Fisher Scientific, Inc.).

Table I. Primers used for targets amplification.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<tbody>
<tr>
<td>SOCS5</td>
<td>AATAAGTGGGATCTGTTCGC</td>
<td>TCCTCCGTTGCAAGTCC-3</td>
</tr>
<tr>
<td>SOCS6</td>
<td>CGGAATTCATGAAACTACGTGAA</td>
<td>CGGAATTCATGAAACTACGTGAA</td>
</tr>
<tr>
<td>SOCS7</td>
<td>CTCTCAGGAGGGCTCCTTC</td>
<td>AAGGCTGGCTGCAAAGAGTGC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>AAGCGATTGCCAATGACAC</td>
<td>AAGCGATTGCCAATGACAC</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>AACTCCAGGGAGCTTGTTTC</td>
<td>CAATGGAACCCGGCTATTCC</td>
</tr>
<tr>
<td>Vimentin</td>
<td>TCAAGGTGCCTGACCTTCTC</td>
<td>TCAACGGCAAGTTCTTCTTTC</td>
</tr>
<tr>
<td>Snail</td>
<td>TTCAACTGCAAATCTGCAACAG</td>
<td>CTGTGGCCCTGGAGTGGTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGATGACATCAAGAAGTGG</td>
<td>TTACTCCTTGGAGCCATGT</td>
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SOCS, suppressor of cytokine signaling.

Luciferase reporter analysis. Vectors of SOCS5-3' untranslated region (UTR), SOCS6-3' UTR, SOCS7-3' UTR, miR-885-5p inhibitor and scramble were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The pmirGLO-3' UTR plasmids SOCS5/SOCS6/SOCS7-WT (containing the wild-type SOCS5/SOCS6/SOCS7 putative 3'-UTR-binding site) and SOCS5/SOCS6/SOCS7-Mut (containing the mutant SOCS5/SOCS6/SOCS7 3'-UTR; Sangon Biotech Co., Ltd.) were constructed. Then the cells were cotransfected with either scramble or miR-885-5p mimics and 3'-UTR-WT or Mut using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega Corporation) 48 h after cell transfection. At 48 h post-transfection, the relative reporter activity was normalized by Renilla luciferase activity.

Expression of miR-885-5p, and SOCS in colorectal cancer tissue and cell line. The relative expression levels of miR-885-5p in colorectal cancer tissues and cells were determined by RT-qPCR, which are shown in Fig. 1A and B. It was demonstrated that the relative mRNA level of miR-885-5p increased significantly in tumor tissues and cells compared with the normal tissues and cells, respectively (P<0.01). Additionally, the mRNA and protein expression levels of three SOCS factors (SOCS5, SOCS6 and SOCS7) in tumor tissues and cells were also detected (Fig. 1C-F). The mRNA levels were significantly lower in tumor tissues and cells compared with that in normal tissues and cells, respectively (P<0.05; Fig. 1C and E). This was supported by the differences in protein levels observed in the western blot analysis, which were also markedly decreased (Fig. 1D and F). Furthermore, correlation analysis revealed that there was a negative correlation between the expression level of miR-885-5p and each SOCS factor (all P<0.0001; Fig. 1G).

miR-885-5p suppression inhibits cell proliferation. SW480 cells were successfully transfected with miR-885-5p inhibitor, mimic and scramble, as demonstrated in Fig. 2A. The MTT assay demonstrated that miR-885-5p suppression significantly inhibited cell proliferation compared to the scramble group (P<0.05; Fig. 2B). In addition, the clonogenic assay showed that the number of colonies in the miR-885-5p inhibitor group was significantly less compared with the scramble group (P<0.05; Fig. 2C and D), which was in accordance with the findings of cell proliferation assay.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). All data are expressed as the mean ± standard error. Tukey's test was used to calculate the difference between two groups. One way analysis of variance followed by Dunnett's post hoc test was used to calculate the difference for more than three groups. P<0.05 was considered to indicate a statistically significant difference.

Results
miR-885-5p suppression inhibits cell migration by regulating epithelial-mesenchymal transition (EMT). The Transwell assay results presented in Fig. 3A and B revealed that the number of migrated cells increased significantly in miR-885-5p mimic group and decreased significantly in miR-885-5p inhibitor group compared with that in miR-885-5p scramble, and control groups (P<0.01). It is well known that EMT serves an important role in tumor migration and invasion; therefore, the expressions of EMT-related proteins, N-cadherin, E-cadherin, vimentin and Snail were detected. As shown in Fig. 3C, when miR-885-5p was suppressed, the mRNA expression levels of E-cadherin increased significantly (P<0.01), while the expression levels of N-cadherin, vimentin and Snail decreased significantly compared with control (P<0.05). This was also supported by the observations in protein expression levels following western blot analysis (Fig. 3D).

miR-885-5p directly regulates SOCS expression. Using TargetScan software (www.targetscan.org), three SOCS target genes (SOCS5, SOCS6 and SOCS7) were predicted, as presented in Fig. 4A. After luciferase reporter analysis of the relative luciferase activities of the three genes, it was revealed that the relative luciferase activity of the reporter that contained the wild-type 3'-UTR of the three SOCS genes reduced significantly in miR-885-5p-inhibitor-transfected cells compared with the control (P<0.05; Fig. 4B). In addition, RT-qPCR and western blot analysis suggested that the relative expression levels of SOCS genes increased when miR-885-5p was suppressed (Fig. 4C and D), indicating that miR-885-5p may directly regulate the SOCS genes.

miR-885-5p suppression inhibits cell migration by targeting SOCS. To further investigate the effect of miR-885-5p on the progression and migration of colorectal cancer from the aspect of molecular mechanisms, si-SOCS was transfected into tumor cells that was transfected with miR-885-5p inhibitor. As presented in Fig. 5A and B, compared with miR-885-5p inhibitor group, the number of migrated cells significantly
increased in the si-SOCS combined with miR-885-5p inhibitor groups (P<0.01). Furthermore, compared with miR-885-5p inhibitor group, the mRNA expression of E-cadherin decreased significantly, and the expression levels of N-cadherin, vimentin and Snail increased significantly in si-SOCS + miR-885-5p inhibitor groups (P<0.05; Fig. 5C). This was validated by the results of the western blotting whereby E-cadherin protein expression markedly decreased, and N-cadherin, vimentin and Snail protein expression levels increased (Fig. 5D). The result suggested that miR-885-5p suppression regulates EMT by targeting SOCS genes, subsequently inhibiting cell migration.
Figure 4. (A) The gene sequences of SOCS5, SOCS6 and SOCS7 regulated by miR-885-5p. (B) The relative luciferase activities in wild-type 3'-UTR of SOCS and mutant SOCS 3'-UTR in transfected cells. The relative expression levels of SOCS5, SOCS6 and SOCS7 in transfected cells assayed by (C) reverse transcription-quantitative polymerase chain reaction and (D) western blot analysis. *P<0.05 and **P<0.01 vs. the control group in C and the miR-control group in B and D, respectively. SOCS, suppressor of cytokine signaling; miR, microRNA; UTR, untranslated region; WT, wild-type; Mut, mutant.

Figure 5. (A) Quantification and (B) representative image of the Transwell migration assay on the effects of miR-885-5p suppression combined with SOCS suppression in colorectal cancer cells. Original magnification, x200. The relative expression levels of N-cadherin, E-cadherin, vimentin and Snail after miR-885-5p suppression combined with SOCS suppression assayed by (C) reverse transcription-quantitative polymerase chain reaction and (D) western blot analysis. *P<0.05 and **P<0.01 vs. the control group. SOCS, suppressor of cytokine signaling; miR, microRNA; siRNA, small interfering RNA.
Discussion

The present study demonstrated that miR-885-5p was upregulated, while SOCS5, SOCS6 and SOCS7 were downregulated in colorectal cancer tissues, and cells. Suppression of miR-885-5p in tumor cells was able to significantly inhibit cell proliferation and migration. In particular, luciferase reporter analysis suggested that SOCS5, SOCS6 and SOCS7 were target genes of miR-885-5p. The findings in the current study may provide novel insights into the molecular mechanisms underlying colorectal cancer.

Novel biomarkers are necessary in clinical to improve the diagnosis and management of diseases. Recent studies have exposed miRNAs as potential biomarkers for several disease conditions, including human cancer types (16-18). miR-885-5p expression has been reported to be dysregulated in several human cancer types, including liver cancer, neuroblastoma and oncocytic follicular thyroid carcinomas (19-21). For instance, in the study by Afanasyeva et al (20), miR-885-5p acts as a tumor suppressor in neuroblastoma through interfering with cell cycle progression and cell survival. In the study by Dettmer et al (21), miR-885-5p was identified to be upregulated in oncocytic follicular carcinoma. In accordance with the findings of Dettmer et al (21), the present study also observed significantly elevated mRNA expression of miR-885-5p in colorectal cancer. To the best of our best knowledge, this is the first study identify an association between the role of miR-885-5p and colorectal cancer.

The current study also investigated the effects of miR-885-5p on cell proliferation and migration of colorectal cancer by inhibiting the expression level of miR-885-5p in SW480 cells. The result showed that cell proliferation and migration significantly decreased following miR-885-5p suppression. Previous studies have revealed that EMT is one of the key molecular steps in the process of distant metastasis, and influences the invasiveness and migratory capacity of cancer, including colorectal cancer (22-24). EMT is a complex process involving loss the apico-basolateral polarity and disruption of cell-cell junctions, subsequently leading to the formation of migratory mesenchymal cells with invasive properties (11). During the process of EMT, cancer cells increase expression of mesenchymal markers, such as N-cadherin and vimentin, while reducing the expression of cellular adhesion proteins, including E-cadherin (25). In addition to E-cadherin, N-cadherin and vimentin, Snail is also considered to contribute to the EMT process (26). The present study investigated the expression levels of four EMT-related proteins, N-cadherin, E-cadherin, vimentin and Snail following miR-885-5p suppression/overexpression, and found that the mRNA expression levels of E-cadherin increased significantly, while the expression of the other proteins decreased when miR-885-5p was suppressed. These findings further demonstrated the inhibiting effect of miR-885-5p suppression on tumor cell migration in colorectal cancer.

In addition to EMT-related proteins, SOCS proteins have also been implicated in regulating cell proliferation and migration in cancer (27,28). In the current study, three SOCS proteins (SOCS5, SOCS6 and SOCS7) were predicted based on the TargetScan software. It was revealed that SOCS5, SOCS6 and SOCS7 were downregulated in colorectal cancer tissues and cells, and were negatively correlated with the expression of miR-885-5p. In addition, luciferase reporter analysis suggested that SOCS5, SOCS6 and SOCS7 were target genes of miR-885-5p. SOCS proteins are induced by cytokines and involved in inhibiting the Janus kinase-signal transducer and activator of transcription (STAT) signaling pathway (29). The SOCS family has eight members, including cytokine-inducible SH2-domain-containing protein and suppressors of cytokine signaling 1-7 (SOCS1-7), which have been investigated in several malignant diseases (30). Alterations in expression of SOCS proteins have been demonstrated in several cancer types, including liver, squamous head and neck, and lung cancer (28,30-32). Importantly, Fujitake et al (33) reported aberrant expression of SOCS1 in patients with colorectal cancer. A previous study also demonstrated that EMT is regulated by STAT1/3 signaling, while SOCS is a negative regulator of STAT1/3 signaling (34). Thus, EMT may be negatively regulated by SOCS, which was in accordance with the present study, whereby the expression of E-cadherin decreased significantly and the expression levels of N-cadherin, vimentin and Snail increased significantly in si-SOCS + miR-885-5p inhibitor groups compared with the miR-885-5p inhibitor group alone.

In conclusion, the present study suggested that the expression of miR-885-5p was upregulated in colorectal cancer. miR-885-5p suppression was able to inhibit cell proliferation and migration, and EMT processes by targeting SOCS5, SOCS6 and SOCS7 genes in colorectal cancer. Therefore, targeting miR-885-5p and SOCS expression may be used for the diagnosis, and treatment of colorectal cancer.

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Competing interests

The authors declare that they have no competing interests.

References


