miR-200b-3p inhibits proliferation and induces apoptosis in colorectal cancer by targeting Wnt1

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Abstract. MicroRNA (miR)-200b-3p is downregulated in multiple human cancer types. Wnt signaling serves a role in human colorectal cancer (CRC). The present study aimed to examine the effect of miR-200b-3p on human CRC and its potential association with Wnt signaling. The Cell Counting Kit-8 (CCK-8) was employed to assess cell viability. A flow cytometric assay was conducted to examine cell proliferation and apoptosis. The regulation model of miR-200b-3p and Wnt1 was assessed by a luciferase reporter assay. A commercial kit was used to evaluate the activity of caspase-3 following treatment of the cells by miR-200b-3p or Wnt1. The expression of target factors was determined by a quantitative real-time polymerase chain reaction and western blot analysis. The expression of miR-200b-3p was decreased in human CRC tissues and in cell lines. The bioinformatics analysis and the luciferase reporter assay revealed that Wnt1 may be a direct target of miR-200b-3p. Moreover, the viability and proliferation of CRC cells was suppressed by miR-200b-3p. miR-200b-3p additionally induced apoptosis in CRC cells. Furthermore, the caspase-3 activity was enhanced in the miR-200b-3p mimics group. The expression of antigen Ki-67 (additionally termed KI-67) and β-catenin was decreased, while the expression of cleaved caspase-3 was increased by miR-200b-3p. In conclusion, miR-200b-3p inhibited proliferation and induced apoptosis in CRC cells by inactivating Wnt/β-catenin signaling. The present study provided potential biomarkers and candidate modalities for the management of CRC.

Introduction

Human colorectal cancer (CRC), one of the most common malignant tumors in China, is a leading cause of tumor-associated mortality worldwide (1-3). The occurrence of CRC is primarily due to age and lifestyle factors (4,5); however, it is additionally a consequence of a number of mutations and alterations in critical oncogenes and suppressor genes (6). Mutations most frequently occur in the Wnt signaling pathway (7). The Wnt signaling cascade is implicated in carcinogenesis (8), embryonic development (9), cell proliferation (10) and apoptosis (11). The canonical Wnt pathway is able to regulate gene transcription, in which β-catenin is involved (12). Thus, this pathway is additionally termed the Wnt/β-catenin signaling pathway. Through the binding of the Wnt protein to the N-terminal extracellular cysteine-rich domain of a Frizzled (Fz) family receptor, Wnt/β-catenin signaling begins and leads to the translocation of accumulated β-catenin into the nucleus, and eventually leads to the transcription of its target genes, including proto-oncogenes (12,13). It has been reported that mutation of β-catenin occurs in 10% of CRC cases (14). Accordingly, factors that may block the activity of Wnt signaling may be modalities for treating human CRC.

MicroRNAs (miRNAs), small non-coding RNAs, were recognized to have regulatory activity in the 2000s (15). Extensive evidence demonstrates that abnormal expression of miRNAs is a key step in tumor progression, in which process miRNAs serve as tumor suppressor genes or oncogenes. miRNAs are able to silence the expression of target mRNAs by binding to the complementary site in the 3′ untranslated region (3′ UTR) (16). It has been claimed that ~60% of genes in humans and other mammals are targets of miRNAs (17). The association of miRNAs and CRC was first identified in 2003 (18). Emerging evidence has demonstrated the role of miRNAs in regulating Wnt signaling, including in CRC (19). Wnt1, an important component of Wnt signaling, may serve as a target gene of miRNAs in multiple cell types (20-22). It has been reported that miRNA (miR)-200b-3p is a member of the miR-200 miRNA family, which is down-regulated in a wide range of human cancer types including CRC (23,24). However, the association between miR-200b-3p and Wnt1 remains unclear.
Thus, the present study sought to examine the effect of miR-200b-3p on the proliferation and apoptosis of human CRC, and to clarify the potential associations between miR-200b-3p and Wnt signaling in the hopes it may provide clues to the mechanism of tumor progression in CRC.

Materials and methods

Tissue specimens. A total of 45 patients (male, mean age 58.8; range 37-75 years) with CRC were enrolled in the present study, with written informed consent. None of the patients received chemotherapy or radiotherapy prior to surgery at Yuhang Branch of Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) between April 2013 and June 2015. Patients who were histologically diagnosed with CRC and had complete clinicopathological data were included. Exclusion criteria included patients who had familial cancer syndrome and inflammatory bowel disease, a history of malignancy in other sites or infectious diseases. The CRC tissues and paracarcinoma tissues (≤5 cm distance from the tumor site) were obtained during the surgery. All the protocols in the present study were approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine.

Cell lines and cell culture. Human CRC cell lines (HCT-8, SW480, LOVO, HT29 and SW620) and a colonic epithelial cell line (NCM460) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in the presence of 5% CO₂.

Cell transfection and grouping. The cells were seeded at a density of 4x10⁵ cells/well in a 24-well plate. When cells reached 50% confluence, the human miR-200b-3p mimics (10 nM; UAAUAC UGCCCUGUAUGAUGA cat. no. HMI0352; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), Wnt-1-small interfering (si)RNA (800 ng/µl; cat. no. HSH059152-CH1; GeneCopeia, Inc., Rockville, MD, USA), si-negative control (800 ng/µl; cat. no. CSHCTR001-CH1; GeneCopeia, Inc.) or miR-control (10 nM; cat. no. HMC0002; Sigma-Aldrich; Merck KGaA) was transfected using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. Following transfection for 48 h, the cells were collected for subsequent experiments. The experimental design was as follows: Control group (untreated cells, mock); negative control group (cells that were transfected with the siRNA negative control, NC); mimics group (cells that were transfected with miR-200b-3p mimics); and si-WNT1 group (cells that were transfected with si-WNT1). All the experiments were independently conducted at least three times.

Cell Counting Kit-8 (CCK-8) assay. The cells were seeded at a density of 1x10⁵ cells/well in a 96-well plate following the aforementioned treatment. The cell viability was detected at different time points (12, 24 and 48 h post-siRNA transfection, respectively). CCK-8 solutions (10 µl) from the CCK-8 kit (cat. no. C0038; Beyotime Institute of Biotechnology, Haimen, China) were supplemented into the wells and incubated for 4 h. The absorbance of the reaction mixtures in each well was detected at 450 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Luciferase reporter assay. The bioinformatics analysis was performed using TargetScan (http://www.targetscan.org/vert_71/) and miRNA.org (http://34.236.212.39/microrna/home.do). The pLightSwitch-Wnt-3’UTR marine RenSP luciferase reporter (cat. no. s812430) and Cypridina TK control vector (pTK-CLuc Vector) (cat. no. 32036) were purchased from Active Motif (Shanghai, China). The corresponding mutation vector was constructed as p-mut-Wnt-3’UTR (CAGUAUU mutated to TACUCUC) using the MutBEST kit (Takara Bio, Inc., Otsu, Japan; cat. no. R401). The cells were plated in a 6-well plate (1x10⁵ cells/well) and cultured overnight. Using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), the Cypridina TK control vector was co-transfected with pLightSwitch-Wnt-3’UTR vector or p-mut-Wnt-3’UTR vector into cells, respectively. Following 48 h, the luciferase activity was measured with a LightSwitch™ Dual Assay kit (Active Motif; cat. no. 32035) on a microplate reader (Bio-Rad Laboratories, Inc.). The Cypridina luciferase activity served as the control.

Caspase-3 activity. The cells in each group were harvested and re-suspended in lysis buffer provided by a caspase-3 assay kit (R&D systems, Inc., Minneapolis, MN, USA; cat. no. BF3100). Following centrifugation at 600 x g for 5 min at room temperature, the supernatant was mixed with reaction buffer and caspase-3 substrate in a 96-well plate, which was incubated for 2 h at 37°C. The absorbance at 405 nm was read with a microplate reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.)
Western blot analysis. Total protein from the cells was isolated using radioimmunoprecipitation assay buffer with protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Proteins from tissue were isolated by a mechanical homogenizer for 10 min, 42˚C for 50 min and 70˚C for 15 min. The concentration and the integrity were determined using a Nanodrop (Thermo Fisher Scientific, Inc.). cDNA was synthesized using 1 µg RNA with the PrimeScript RT Reagent kit (Takara Bio Inc.), according to the manufacturer's protocol. SYBR-Green PCR (Takara Bio Inc.) was used to perform the qPCR on the ABI 7500HT System (Thermo Fisher Scientific, Inc.). The primer pairs used were as follows: Wnt1 (forward), 5'-TGG CTTGTTTCTCTGCTAGC-3'; Wnt1 (reverse), 5'-CCCGGA TTGGGCGTATC-3'; miR-200b (forward), 5'-GCCGCTAAAT ACTGCTCTGTTA-3'; miR-200b (reverse), 5'-GTGCAGGGTG CCGAGGT-3'; Ki67 (forward), 5'-ACTGCAGCAGTGGAG ACTAGG-3'; Ki67 (reverse), 5'-AGAACAGTAGCCTGATGT TTGG-3'; β-actin (forward), 5'-GATGAGTGTCGCAAGG ATTCCT-3'; β-actin (reverse), 5'-TGTCCTCAGTTGGTG ACGAT-3'; β-actin (forward), 5'-GCTGAGAAGCGGGAAG CCTGT-3'; β-actin (reverse), 5'-GCCAGGGTGCTAAG CAG-3'; U6 (forward), 5'-CTCGTCTCGGCCAGACA-3'; and U6 (reverse), 5'-AACGCTTCAAGATTTGCGT-3'. The reactions were conducted using the following thermocycling conditions: Initial denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 15 sec, and 60˚C for 1 min; 72˚C for 10 min for extension. The expression of target genes was calculated using the 2^−ΔΔCq method (25). The expression fold change was relative to the internal housekeeping genes β-actin and U6.

Results

Expression of miR-200b-3p and Wnt1 in CRC tissues. The dysregulation of miR-200b-3p and Wnt1 has been reported in tumorigenesis (26,27). Thus, the expression of miR-200b-3p and Wnt1 in patients with CRC was investigated. According to the RT-qPCR results from carcinoma tissues and para-carcinoma tissues, it is noteworthy that the expression of miR-200b-3p was significantly decreased in tumor tissues compared with that in para-carcinoma tissues (P<0.001; Fig. 1A). By contrast, the expression of Wnt1 was significantly increased in tumor tissues compared with that in normal tissues (P<0.001; Fig. 1B). Moreover, the expression of miR-200b-3p and Wnt1 was negatively correlated in CRC (r=-0.32; P=0.031; Fig. 1C). In addition, six cases were randomly selected to determine the expression of Wnt1 at the translational level. It was demonstrated that the expression of Wnt1 was increased in tumor tissues compared with that in normal tissues (Fig. 1D), with the exception that there was no significant difference in the fourth case (P=0.01; Fig. 1E).

Expression of miR-200b-3p and Wnt1 in CRC cell lines. Subsequently, the expression of miR-200b-3p and Wnt1 was examined in different CRC lines including HCT-8, SW480, LOVO, HT29 and SW620. The colonic epithelial cell line NCM460 was used as a control. The results revealed that the expression of miR-200b-3p was decreased in all these CRC lines (Fig. 2A); whereas, the expression of Wnt1 was increased at the transcriptional and the translational level (Fig. 2B-D). The most marked alteration in the expression of miR-200b-3p and the expression of Wnt1 was observed in HCT-8. Thus, HCT-8 was selected for the subsequent experiments.

Wnt1 may be a direct target of miR-200b-3p. It is widely accepted that miRNAs exert function by silencing target mRNAs (28). Therefore, a bioinformatics analysis was performed using TargetScan (http://www.targetscan.org/vert_71/) and miRNA.org (http://34.236.212.39/microrna/home.do). The data from the two databases displayed that there was a potential binding site for miR-200b-3p in the 3' UTR of the Wnt1 mRNA sequence (Fig. 3A). To verify this prediction, a luciferase reporter assay was conducted. It was revealed that the luciferase activity of wild-type Wnt1-3'UTR was significantly
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Effect of miR-200b-3p on the cell viability, proliferation and apoptosis of CRC cells. The effect of miR-200b-3p on CRC cells was detected with regards to cell viability, cell proliferation and apoptosis. The cell viability assay revealed that the Wnt1 interference suppressed the viability of CRC cells significantly, beginning at 24 h (P<0.05; Fig. 4A). By contrast, the significant decline in cell viability in the miR-200b-3p mimics group was observed following transfection for 48 h (P<0.05; Fig. 4A). In addition, the transfection efficiency of miR-200b-3p and Wnt1 was verified, respectively (Fig. 4B-E). Furthermore, the results from the CFSE assay revealed that cell proliferation was significantly inhibited in the miR-200b-3p mimics group and si-Wnt1 group compared with that in the control group, respectively (P<0.01; Fig. 5A). By contrast, the apoptosis level was significantly higher with the presence of miR-200b-3p mimics and si-Wnt1 compared with that in the control group, respectively (P<0.01; Fig. 5B).

Effect of miR-200b-3p on the expression of Ki67, cleaved caspase-3 and β-catenin. Caspase-3 is an important protein implicated in multiple apoptosis pathways (29), while Ki67 is recognized to be a cell proliferation-associated protein (30). As an important component of the canonical Wnt pathway,
β-catenin is dysregulated in numerous carcinomas, including CRC (31); thus, the effect of miR-200b-3p on their expression was further examined. It was demonstrated that caspase-3 activity was elevated significantly in the miR-200b-3p mimics group and the si-Wnt1 group compared with that in the control group, respectively (P<0.01; Fig. 6A). Expression of Ki67 was reduced in the miR-200b-3p mimics group; the expression of Ki67 and β-catenin was decreased in si-Wnt1 group compared with that in the control group (Fig. 6B). Furthermore, the protein level of pro-caspase-3 and cleaved caspase-3 was decreased and increased in the miR-200b-3p mimics group and the si-Wnt1 group compared with that in the control group, respectively (Fig. 6C-E).

**Discussion**

The present study illustrated the role of miR-200b-3p in the proliferation and apoptosis of CRC, and highlighted the importance of increased expression of Wnt1 in CRC progression.
In the present study, miR-200b-3p was frequently decreased in the CRC tissues and cell lines, compared with the paracarcinoma tissues or normal cells. Decreased expression of miR-200b has been identified to be closely associated with poor survival in ovarian carcinomas (32). Another study also indicated that miR-200b may be a prognostic marker for breast cancer (33). These results indicated the potential of miR-200b as a valuable biomarker for tumors. Wnt1, as an important ligand involved in Wnt/β-catenin signaling (27), is generally upregulated in human cancer (34). Similarly, the expression of Wnt1 was elevated in the CRC tissues and cell lines in the present study. Furthermore, the protein expression of Wnt1 was increased in approximately all tumor tissues from patients with CRC. Additionally, the expression of miR-200b-3p and Wnt1 was negatively correlated. It was indicated that decreased miR-200b-3p and elevated Wnt1 expression may be critical in the progression of CRC.

In consideration of the regulatory machinery of miRNAs, it was proposed that the expression of Wnt1 may be downregulated by miR-200b-3p. Thus, bioinformatics analysis was undertaken to confirm this hypothesis. According to the data from available online databases, it was revealed that there was a complementary sequence of miR-200b-3p in the 3'UTR of Wnt1. Subsequently, a luciferase reporter assay was performed to confirm this prediction. The presented data revealed that the luciferase activity of wild-type Wnt1-3'UTR was reduced by miR-200b-3p mimics, whereas the activity of mutant Wnt1-3'UTR was not affected. It was indicated that Wnt1 may be a direct target of miR-200b-3p. Therefore, there was further speculation that miR-200b-3p may be a tumor suppressor of CRC by regulating Wnt signaling.

To confirm the effect of miR-200b-3p on CRC and the underlying mechanism, the proliferation and apoptosis behavior of CRC following miR-200b-3p transfection was examined. It was demonstrated that the viability of CRC cells and CSFE fluorescence intensity was decreased via miR-200b-3p. Cellular apoptosis in CRC was increased in the miR-200b-3p mimics group compared with the control group.
A previous study reported that miR-200b-3p may inhibit the metastasis of prostate cancer (26). The data presented in the present study combined with previous results from another study demonstrated the tumor suppressive ability of miR-200b-3p (35).

Sequential activation of caspases is the hallmark event in cellular apoptosis. Caspase-3, as an executioner caspase, is at the convergence of multiple apoptosis pathways (29,36,37). Furthermore, Ki67, as a strictly cell proliferation-associated factor (30), is present in all active phases in cell cycle progression and is inactive in the G0 phase (38). The activity of these molecules, which are associated with apoptosis and cell proliferation, was investigated. The present data demonstrated that the activity of caspase-3 was enhanced by miR-200b-3p. The expression of Ki67 was deduced and cleaved caspase-3 was enhanced in the presence of miR-200b-3p mimics. Furthermore, the abnormal activation of Wnt/β-catenin signaling is frequently identified in CRC (39,40). Therefore, the effect of miR-200b-3p on the expression of β-catenin was examined in the present study.

Figure 5. Effect of miR-200b-3p on the proliferation and apoptosis of CRC cells. (A) CFSE assay for cell proliferation. (B) Flow cytometric analysis for apoptosis. *P<0.01 vs. mock group. siRNA-NC, small interfering RNA negative control; si-Wnt1, small interfering RNA-Wnt1; CRC, colorectal cancer; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; miR, microRNA.
The protein expression level of β-catenin was decreased via miR-200b-3p, although its mRNA expression was reduced slightly. It was suggested that miR-200b-3p decreased the activity of β-catenin. In addition, the inactive status of Wnt signaling may lead to the degradation of β-catenin (41). Thus, the decreased β-catenin protein expression level may be in part due to the silencing of Wnt1 that was mediated through miR-200b-3p. Taken together, it was suggested that miR-200b-3p may suppress the tumor progression of CRC by inhibiting Wnt/β-catenin signaling. The present study proposed that Wnt1 and miR-200b-3p may serve as potential biomarkers for predicting the occurrence of CRC and candidate modalities for targeted therapy for CRC.

A limitation of the present study was that the direct binding of miR-200b-3p to Wnt1 requires validation by in-depth investigations. A previous study reported that histone deacetylase 1/4 and specificity protein 1 (Sp1) may regulate the miR-200b expression, thereby affecting chemoresistance and epithelial-mesenchymal transition, respectively (42,43). However, another previous study claimed that Sp1 was a target of miR-200b (33). Thus, future examination of the upstream regulator may be interesting and valuable for understanding the molecular mechanisms underlying the progression of CRC. In addition, the miR-200 family has been demonstrated to have an anti-tumor effect (44). Thus, determining the combined effect of miR-200b-3p and other family members on CRC may inspire another promising strategy for the treatment of CRC.

In conclusion, the present study uncovered valuable clues underpinning the mechanism of tumor progression in CRC. The present data revealed that miR-200b-3p and Wnt1 serve important roles in the progression of CRC cells. Wnt1 may be a direct target of miR-200b-3p. Furthermore, miR-200b-3p suppressed proliferation and induced apoptosis in CRC by regulating the activity of caspase-3, Ki67 and β-catenin. These results may have clinical implications for patients with CRC.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors’ contributions

LC wrote the main manuscript. LC, XW, YZ and JZ performed the experiments. LC and QL designed the study. XW and YZ performed data analysis. All authors reviewed the manuscript.

Ethics approval and consent to participate

All the protocols in the present study were approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine and written informed consent was obtained.

Consent for publication

Informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References


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