MicroRNA-155 enhances the activation of Wnt/β-catenin signaling in colorectal carcinoma by suppressing HMG-box transcription factor 1

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Abstract. Colorectal carcinoma (CRC) is a malignant solid tumor arising from the large intestine and is associated with an increasing incidence and poor prognosis. Further understanding of the molecular mechanisms underlying CRC may contribute to the development of novel effective therapeutic strategies. MicroRNAs (miRs), including miR-155, have been reported to be associated with the etiology and biology of CRC; however, the molecular mechanisms by which miR-155 affect CRC remain to be fully elucidated. The present study used a multidisciplinary approach, involving reverse transcription-quantitative polymerase chain reaction, northern blotting, MTT assay, cell cycle progression analysis, immunoblotting, and animal experiments, to determine the possible targets of miR-155 in CRC cells. miR-155 was found to be overexpressed in CRC tissue samples, compared with paired normal colon tissue samples. In addition, the inhibition of miR-155 induced a deceleration in CRC cell proliferation and inactivation of the Wnt/β-catenin signaling pathway. Furthermore, patients with CRC exhibiting higher serum levels of miR-155 exhibited reduced survival rates. In conclusion, the present study demonstrated that miR-155 may contribute to the progression and growth of CRC by enhancing the Wnt/β-catenin pathway in an HBP1-associated mechanism. Therefore, miR-155 may be considered a promising therapeutic target for the treatment of CRC.

Introduction

Colorectal carcinoma (CRC) is a common type of cancer in developed and developing countries (1). CRC is refractory to current therapeutic strategies, due to the lack of knowledge regarding the molecular mechanisms underlying CRC (2). Therefore, it is important to examine the molecular mechanism associated with the initiation, progression, invasion and recurrence of CRC.

Increasing evidence has indicated that microRNAs (miRNAs) are important in the regulation and abundance of target mRNAs. Mechanistically, miRNAs recognize and bind the corresponding miRNA recognition elements (MREs) within the 3′-untranslated region (UTR) of target mRNAs, and the affected mRNA undergoes degradation or translational inhibition (3). The involvement of miRNAs in cancer has been the focus of previous studies (4,5). miR-155 is an oncogenic miRNA in several types of cancer, which has been demonstrated to promote the initiation and progression of malignant tumors (5). In addition, the diagnostic and prognostic value of miR-155 has been verified in previous studies (6-8).

miR-155 is associated with CRC, and high expression levels of miR-155 have been detected in CRC specimens, which is significantly correlated with lymph node metastases (5). The overall survival and disease-free survival rates of patients with high expression levels of miR-155 are significantly reduced, compared with those of patients with low expression levels of miR-155 (7). Zhang et al (8) reported that miR-155 promotes the migration and invasion of CRC cells, possibly by regulating the expression of claudin-1. In addition to its role in existing CRC tumors, miR-155 may also contribute to the initiation of CRC. For example, miR-155 has been reported to be overexpressed in inflammatory bowel disease (9) and ulcerative colitis (10), which are closely associated with CRC.

Abbreviations: CRC, colorectal carcinoma; HBP1, HMG-box transcription factor 1; MRE, miRNA recognition elements; siRNA, small interfering RNA; siHBP1, HBP1 siRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution

Key words: MicroRNA-155, HMG-box transcription factor 1, colorectal carcinoma, Wnt
miR-155 may be an effective therapeutic target, as isothiocyanates have been demonstrated to suppress the tumorigenesis of CRC by decreasing levels of miR-155 levels (11). However, the molecular mechanism underlying the effects of miR-155 on CRC remain to be fully elucidated.

Therefore, the present study used CRC cell lines and a CRC xenograft murine model to investigate the molecular pathways affected by miR-155.

Materials and methods

Cell culture. Four human CRC cell lines: SW480, SW620, HT-29 and HCT-116, and one normal colon epithelial cell line (CCD-18Co), were purchased from American Type Culture Collection (Manassas, VA, USA). The HEK-293T normal human embryonic kidney cell line was obtained from Shanghai Cell Collection (Shanghai, China). The above cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine at 37°C in an atmosphere containing 5% CO2.

CRC specimens. CRC tissue and adjacent normal colonic mucosa tissue samples were obtained by surgical removal from patients diagnosed with primary CRC (eight men and seven women; mean age, 53.4 years) at the Department of Gastrointestinal Surgery, China-Japan Union Hospital, Jilin University (Changchun, China). The patients provided written informed consent and the present study was approved by the ethics committee of the China-Japan Union Hospital, Jilin University. The tumor samples (average size, 2.23 cm) and adjacent non-cancerous tissue samples (average size, 1.1 cm) were placed in liquid nitrogen and then stored at 280°C.

Formalin-fixed and paraffin-embedded tissues were cut into 4 µm sections.

qPCR was performed using an All-in-One™ miRNA qRT-PCR Detection kit (AOMD-Q020; GeneCopoeia) and a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). U6 was selected as an endogenous control. The primers and probes for miR-155 and U6 were obtained from GeneCopoeia.

In order to detect the mRNA expression levels of Axin2, CD44 and Lgr5, the isolated RNA was transcribed into cDNA using a ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions, followed by a qPCR assay. Total RNA was polyadenylated using a Poly(A) Polymerase Tailing kit (Epicentre, Chicago, IL, USA). The reaction mixture included 1 mg RNA, 1 ml of 10 mM ATP, 1 ml of 10X reaction buffer, and 1 U Poly(A) polymerase. The reaction was conducted by incubation at 37°C for 25 min followed by enzyme inactivation at 65°C for 6 min. Reverse transcription was subsequently performed with a reaction mixture containing 1 ml polyadenylation reaction product, 1 ml AMV 10X reaction buffer, 1 ml of 0.5 mM RT primer, 0.5 ml of 10 mM dNTP, and 50 U of AMV High Performance Reverse Transcriptase. The reaction was conducted with incubation at 42°C for 45 min, followed by 70°C for 10 min. The PCR reaction was performed at 95°C for 30 sec, followed by 36 cycles of 95°C for 10 sec and 60°C for 30 sec. cDNA was mixed with primers and SYBR® Green PCR Master Mix according to the manufacturer's protocol. Quantification was performed using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The primer sequences were as follows: Axin2, forward 5’-CCCGTGACCAAGTCTCATC-3’ and reverse 5’-TGGCAGGGCAACAGA-3’; CD44, forward 5’-AGTTCC TGGATCACCGCAGAC-3’ and reverse 5’-GGTCTCTGTGGC-3’; Lgr5, forward 5’-TGAACACCTGCTGGTGA TGCT-3’ and reverse 5’-TGTGGCAGCATGACCCCAATTA-3’; HMG-box transcription factor 1 (HBP1), forward 5’-TGGATCTCTGTGGC-3’ and reverse 5’-GCCCTCTGAGTTCGACC-3’. The blots were subsequently transferred onto nylon membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA) and subjected to ultraviolet cross-linking. The blots were subsequently transferred onto nylon membranes (GE Healthcare Life Sciences, Little Chalfont, UK), and subjected to ultraviolet cross-linking. The blots were hybridized with digoxigenin (DIG)-labeled miR-155 or U6 probes (Exiqon, Vedbaek, Denmark) overnight at 4°C. The membranes were then washed using a low-stringency buffer containing Tris-Hcl 20 mM, NaCl 150 mM, CaCl2

Northern blotting. Northern blot analysis was performed to determine the levels of miR-155 in the indicated samples and cell lines, according to previously described procedures (13). Briefly, total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which was then separated using 15% urea-PAGE (8 M; 480 g urea/liter; Sigma-Aldrich). The blots were subsequently transferred onto nylon membranes (GE Healthcare Life Sciences, Little Chalfont, UK), and subjected to ultraviolet cross-linking. The blots were hybridized with digoxigenin (DIG)-labeled miR-155 or U6 probes (Exiqon, Vedbaek, Denmark) overnight at 4°C. The membranes were then washed using a low-stringency buffer containing Tris-Hcl 20 mM, NaCl 150 mM, CaCl2
10 mM, 1% Triton x-100 (Sigma-Aldrich). A DIG Luminescent Detection kit (Roche Diagnostics, Basel, Switzerland) was used to examine the abundance of miR-155, according to the manufacturer’s protocol.

miR-155 inhibitor and mimic treatment of cells. To alter the expression levels of miR-155 in the cells, mirVana™ miRNA inhibitors or mimics (30 nM) (Invitrogen Life Technologies) were transfected into SW620, HT-29, CCD-18Co and HEK-293T cells using Lipofectamine® 2000, according to the manufacturer’s instructions, 48 h prior to subsequent experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The SW620 and HT-29 cells were plated at a density of 5x10^3 cells into 96-well plates. After 6 h at 37˚C, the cells were treated with the indicated mimics/inhibitor. After 0, 24, 48 or 72 h, the cells were treated with 10 µl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37˚C. Following treatment, the medium containing the MTT solution was removed and 150 µl dimethyl sulfoxide was added. Absorbance was then spectrophotometrically determined at a wavelength of 570 nm using a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Inc.). Cell viability was calculated as follows: Viability (%) = absorbance value of adenovirus-infected cells / absorbance value of control cells.

Cell cycle progression analysis. Cell cycle analysis was performed according to previously described methods (14).

Briefly, 2x10^5 cells were fixed with cold 70% ethanol for 1 h at 48 h following the treatments described above. R Nase A (1%; Sigma-Aldrich) was then used to remove the contaminated RNA. Propidium iodide (50 mg/ml; Sigma-Aldrich) was added to the fixed cells, which were then subjected to flow cytometry (FACSAria™ II; BD Biosciences, Franklin Lakes, NJ, USA). The proportion of cells in the G0/G1, S and G2/M phases were determined using Modfit software 2.0 (BD Biosciences).

Immunoblot assay. An immunoblot assay was performed, according to routine laboratory procedures. Briefly, all of the cells were lysed using radioimmunoprecipitation assay buffer containing 1 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride (Sigma-Aldrich) for 45 min. Subsequently, 45 µg of each total protein was separated by 10% SDS-PAGE prior to being transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated in 1% bovine serum albumin (Sigma-Aldrich) for 4 h at room temperature. The membranes were then washed three times with Tris-buffered saline with Tween 20 (TBST; Sigma-Aldrich). The membranes were incubated with the following primary antibodies at 4˚C overnight at a dilution of 1:1,000: Monoclonal rabbit anti-human Ki-67 (cat. no. 9129), total β-catenin (cat. no. 9582), phosphorylated β-catenin (cat. no. 2009) and GAPDH (cat. no. 5174) (Cell Signaling Technology, Inc.; Danvers, MA, USA) and HBPI (cat. no. ab83402; Abcam, Cambridge, UK). Then the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h following three washes with TBST.
The expression levels were quantified using a Tanon GIS Gel Imager system (Tiangen, Beijing, China).

Animal experiments. The animal experiments performed in the present study were approved by the Committee on the Use and Care of Animals of the China-Japan Union Hospital, Jilin University. A SW620 CRC xenograft was established by injecting 4x10^6 cells into the flanks of 4-7 week-old male BALB/c nude mice (n=14) obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). The mice were housed at 26°C with 10 h light each day, and provided access to sterile food and water with separate feeding. The 14 mice were randomly divided into two groups (n=7), and six of those were chosen for subsequent experimentation. Once the diameter of the tumor reached 5-9 mm, miR-155 inhibitors or control molecules were intratumorally...
Figure 4. miR-155 suppression impairs activation of the Wnt/β-catenin pathway. (A) mRNA expression levels of Axin2, CD44 and Lgr5 were evaluated in colorectal carcinoma cells transfected with miR-155 inhibitor using reverse-transcription-quantitative polymerase chain reaction. (B) A TOPflash assay was used to detect activation of Wnt/β-catenin signaling 48 h following transfection with the miR-155 inhibitor. "P<0.01, vs. the control group. The data are presented as the mean ± standard deviation. (C) Immunoblot analysis of phospho-β-catenin and total β-catenin was also performed. miR, microRNA; phospho-, phosphorylated.

Figure 5. HBP1 is a target of miR-155. (A) HBP1 3'-UTR harboring an miRNA recognition element of miR-155. Protein expression of HBP1 was determined using immunoblot analysis in colorectal carcinoma cells and normal cells transfected with (B) miR-155 inhibitor or (C) mimic. (D) Luciferase activity of cells transfected with pMIR-Reporter-HBP1-3UTR-wt, pMIR-Reporter-HBP1-3UTR-mut and pMIR-Reporter vectors co-transfected with the miR-155 mimic. "P<0.01, vs. the control group. The data are presented as the mean ± standard deviation. HBP1, HMG-box transcription factor 1; miR, microRNA; UTR, untranslated region.
administered into the xenografts (30 nM in 100 µl). The tumor diameters were periodically measured using vernier calipers, and the tumor volume was calculated as follows: Tumor volume (mm$^3$) = [maximal length (mm) x perpendicular width (mm)]$^2$/2.

**TOPflash assay.** A TOPflash assay (Genomeditech) was performed to determine the activation of Wnt/β-catenin signaling, in which a TCF-responsive luciferase-expressing plasmid (Genomeditech, Shanghai, China) was transfected into the SW620 and HT-29 cell lines at a cell density of 70% in 10 cm culture dishes. The transfection was performed at 37°C for 24 h. A luciferase assay was performed, as previously described (15). Briefly, the growth medium was removed from the cells to be assayed. The cells were then washed twice with phosphate-buffered saline, with care so as not to dislodge any of the cells. A minimal volume of 1X Cell Culture Lysis reagent (Promega Corporation, Madison, WI, USA) was added to cover the cells (250 µl for a 60 mm dish), and the cells were incubated for 15-30 min at room temperature. The attached cells were removed from the culture dish and transferred to a microcentrifuge tube. The solution was centrifuged for 5 sec at 10,000 x g to pellet the cell debris. The supernatant (cell extract) was then transferred to a new tube and the pelleted cell debris were discarded. A total of 20 µl cell extract was added to 100 µl luciferase assay reagent (Promega Corporation) at room temperature. The solution was placed in an Lmax II Luminometer (Molecular Devices, Sunnyvale, CA, USA). The light produced was measured for 10 sec and the data recorded.

**Identification of miR-155 targets.** The TargetScan (http://www.targetscan.org/) online database was used to identify potential miR-155 targets, and *HBP1* was identified as a predicted miR-155 target. In order to verify *HBP1* as an authentic miR-155 target, a pMIR-REPORT vector was reconstructed by inserting
a luciferase reporter vector (Genomeditech) containing a 205-bp long DNA sequence of HBP1 3’-UTR with a putative miR-155 binding site (AGCATTAA), in order to generate pMIR-REPORT-HBP1-3UTR-wt (Genomeditech). A luciferase vector harboring a mutant miR-155 binding site (AGCATTAA) was also constructed and used as a control (pMIR-REPORT-HBP1-3UTR-mut). miR-155 mimics were transfected into the HEK-293T cells using Lipofectamine® 2000, and luciferase activity was detected using a Dual-Luciferase® Reporter Assay kit (Promega Corporation).

HBP1 small interfering (si)RNA. HBP1-specific small interfering RNA (siHBP1) was used to reduce the expression levels of HBP1 in the cells. The HBP1 siRNA had the following sequence: forward, 5’-GCUUGACUGUGUAACAGCATT-3’, and reverse, 5’-UGCUGUACCACAGUCAAAGCTT-3’. siHBP1 was obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China), and 10 ng/ml was transfected into the SW-620 cells, together with the miR-155 inhibitors or controls. Following 48 h culture at 37°C, the previously described immunoblot assay was performed.

Statistical analysis. The data were representative of three independent experiments performed in triplicate and were presented as the mean ± standard deviation. The data in the present study were analyzed using a two-tailed Student’s t-test. Statistical analyses were conducted using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of miR-155 are increased in CRC. Initially, the expression levels of miR-155 were elevated in the CRC tissue samples, compared with the paired normal colon tissue samples, as determined by RT-qPCR (n=30; P<0.01; Fig. 1A). Furthermore, in the CRC cell lines, the expression levels of miR-155 were increased (P<0.05; Fig. 1B). Northern blot analysis demonstrated that the miR-155 bands were significantly denser in the CRC tissue samples and cell lines, compared with normal tissue samples and cell line (Fig. 1C).

miR-155 inhibition reduces the proliferation of CRC cells. An miR-155 inhibitor was used to suppress the endogenous expression of miR-155 in the CRC cells (Fig. 2A). Subsequent MTT assays revealed that the proliferation rates of the SW620 and HT-29 cells were suppressed following transfection with miR-155 inhibitor (Fig. 2B). The downregulation of miR-155 also induced an accumulation of cells in the G0/G1 phase (Fig. 2C). In addition, a biomarker of proliferation, Ki-67, was consistently underepressed in the SW620 and HT-29 cells transfected with the miR-155 inhibitor (Fig. 2D).

miR-155 inhibition reduces the growth of CRC xenografts in mice. In a CRC xenograft murine model, treatment with miR-155 inhibitor impaired the growth of established SW620 xenografts, as evidenced by a lower tumor volume in the miR-155-silenced group (Fig. 3A). Furthermore, the weights of the CRC tumors in the mice treated with miR-155 inhibitor were reduced, compared with those in the control group (Fig. 3B).

miR-155 inhibition suppresses activation of the Wnt/β-catenin pathway. The downregulation of miR-155 suppressed the activation of the Wnt/β-catenin pathway, as evidenced by downregulation of Wnt/β-catenin pathway responsive target genes, including Axin2, CD44 and Lgr5 (Fig. 4A) in the cells transfected with a miR-155 inhibitor, and reduced activity of the TOPflash plasmid, which reflects activation of the Wnt/β-catenin signaling pathway (Fig. 4B). However, in the cells transfected with the miR-155 inhibitor, the phosphorylation of β-catenin was not affected (Fig. 4C).

HBP1 is targeted and suppressed by miR-155 in CRC. It was suggested that a potential MRE of miR-155 exists within the 3’-UTR of HBP1 mRNA (Fig. 5A). Inhibition of miR-155 induced an elevation in the protein expression of HBP1, whereas forced overexpression of miR-155 decreased its expression levels (Fig. 5B and C). In the HEK-293T cells, luciferase expression by pMIR-REPORT-HBP1-3UTR-wt was significantly suppressed following transfection with exogenous miR-155, whereas those of pMIR-REPORT-HBP1-3UTR-mut or pMIR-REPORT were unaffected (Fig. 5D).

HBP1 mediates the effects of miR-155 inhibition on CRC cell proliferation and Wnt/β-catenin pathway activation. siHBP1 was used to suppress the expression of HBP1 following miR-155 silencing (Fig. 6A). siHBP1 increased the mRNA expression levels of Axin, CD44 and Lgr5, which were reduced following transfection with the miR-155 inhibitor (Fig. 6B). The TOPflash assay further confirmed that HBP1 suppression restored the activation of the Wnt/β-catenin pathway following transfection with the miR-155 inhibitor (Fig. 6C). Additionally, the MTT assays revealed that the suppression of HBP1 attenuated the effects of miR-155 silencing on CRC cell proliferation (Fig. 6D). In addition, flow cytometric analysis of cell cycle progression demonstrated that siHBP1 reversed G0/G1 arrest in the SW620 cells transfected with the miR-155 inhibitor (Fig. 6E).

These above data indicated that HBP1 was required for the effects of miR-155 on CRC cell proliferation and activation of the Wnt/β-catenin pathway.

High expression levels of miR-155 are associated with reduced survival rates in patients with CRC. Spearman’s rank analysis demonstrated that patients with high levels of miR-155 exhibited a shorter survival rate following surgery, compared with patients exhibiting low levels of miR-155. These results suggested an inverse association between the expression of miR-155 and patient survival rate (Fig. 7).

Discussion

miR-155 has been demonstrated to be overexpressed in CRC cells (7). The results of the present study supported these findings in CRC specimens. The role of miR-155 in CRC has been reported to be associated with enhanced invasion and metastasis (8). The present study also demonstrated that miR-155 promoted the proliferation of CRC cells, suggesting that
targeting miR-155 may be an effective therapeutic strategy to the control the growth of CRC.

During the identification of possible targets of miR-155, HBPI was identified as a gene, which contains a putative miR-155 MRE within its 3′-UTR. Subsequent experiments demonstrated that miR-155-induced suppression of HBPI accounted for its effects on activation of the Wnt/β-catenin signaling pathway. Previous studies have revealed that overexpression of miR-155 promotes activation of the Wnt/β-catenin signaling pathway (16,17). However, these findings were attributed to miR-155-induced activation of Wnt/β-catenin signaling to suppression of adenomatous polyposis coli, a Wnt/β-catenin inhibitor. The results of the present study provided an alternative explanation for the molecular mechanism by which miR-155 affects the Wnt/β-catenin pathway.

The tumor suppressor activity of HBPI has been reported in several types of cancer (18,19). Low expression levels of HBPI result from deregulation of its regulatory systems. For example, methylation of the HBPI promoter has been reported as one of the reasons for the reduced expression of HBPI in lung cancer cells (19). As expected, miRNAs are also involved in the regulatory network of HBPI expression. miR-17-5p and miR-96 have been demonstrated to suppress the expression of HBPI in breast cancer (20) and glioma (21), respectively. The present study indicated that miR-155 may also be an HBPI-regulating miRNA.

In conclusion, the present study demonstrated that miR-155 promoted the growth of CRC in vitro and in vivo, and HBPI was verified as a target gene of miR-155. Therefore, targeting the miR-155/HBPI/Wnt/β-catenin signaling pathway may be an effective therapeutic strategy for the treatment of CRC.

References