miR-203 inhibits cell growth and regulates G_1/S transition by targeting Bmi-1 in myeloma cells

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Abstract. The oncogene B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1) is overexpressed in multiple myeloma (MM). Our previous study demonstrated that Bmi-1 silencing sensitized MM cells to bortezomib. Translational regulation has emerged as a prominent underlying mechanism of Bmi-1 regulation, particularly via microRNA targeting. The present study determined that Bmi-1 may be directly targeted by miR-203 using a luciferase assay. In addition, enforced expression of miR-203 led to significant downregulation of Bmi-1 protein and mRNA expression levels. Furthermore, restoration of miR-203 significantly inhibited cell growth and G_1/S transition in MM cells. miR-203 was downregulated in MM patients, and a negative correlation between the expression of miR-203 and Bmi-1 was observed. The results of the present study indicated that miR-203 exerts growth-inhibiting effects in MM through the suppression of Bmi-1 expression. In conclusion, the present study demonstrated that Bmi-1 is a direct functional target of miR-203 in MM.

Introduction

B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1), a member of the polycomb family, was originally described as interacting with c-Myc to initiate lymphoma in mice (1). Evidence suggests that Bmi-1 is critical for numerous physiological and pathological processes, including axial patterning (2), hematopoiesis (3), regulation of proliferation, senescence (4) and self-renewal of cancer stem cells (5). In addition, Bmi-1 is overexpressed in various malignancies (6-8), including multiple myeloma (MM) (9), and may regulate proliferation and carcinogenesis. Furthermore, our previous study demonstrated that silencing of Bmi-1 sensitizes MM cells to bortezomib (10). Bmi-1 may be post-transcriptionally regulated by microRNAs (miRNAs) (11-14); however, whether miRNAs target Bmi-1 in MM remains unclear.

miRNAs are endogenous non-coding small RNAs that post-transcriptionally regulate gene expression. They exert this effect by binding to the 3'untranslated regions (UTRs) of target mRNAs, resulting in degradation or repression of translation of mRNAs. miRNAs may function as oncogenes or tumor suppressor genes in human cancer (15). Studies have suggested that numerous cancer-associated miRNAs are located in genomic breakpoint regions (16). Certain miRNAs may be epigenetically inactivated, particularly by hypermethylation, and re-expression of these miRNAs may result in the downregulation of target oncogenes (17). Hypermethylation of miR-203 in chronic myeloid leukemia (CML) (18), hepatocellular carcinoma (19) and MM (20) has been described. Restoration of miR-203 downregulates the oncogenic BCR-ABL1 fusion protein and cyclic AMP responsive element binding protein 1 (CREB1) in CML and MM, respectively, thereby inhibiting cellular proliferation and demonstrating the tumor suppressor activity of miR-203. Computational analysis indicates that a single mRNA molecule may be regulated by multiple miRNAs and that one miRNA may regulate multiple mRNAs (21). Therefore, miR-203 may target mRNAs other than CREB1 in MM.

The present study used bioinformatics databases, TargetScan, PicTar and miRanda, to predict the potential miRNAs that may target Bmi-1. A luciferase reporter assay was performed to determine that miR-203 directly targets the 3'UTRs of Bmi-1. Enforced expression of miR-203 significantly inhibits cell growth and regulates G_1/S transition in MM cells. miR-203 was downregulated in MM and the expression of miR-203 was negatively correlated with Bmi-1 expression. Taken together, these results indicated that Bmi-1 is a critical target of the tumor suppressor activity of miR-203 in MM.

Materials and methods

Patient samples. A total of 45 patients with newly diagnosed MM [24 males, 21 females; median age, 55 years (range, 45-75 years)] were recruited from the Department of Hematology, Affiliated Union Hospital of Fujian Medical

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University (Fuzhou, China). The diagnosis of MM was based on standard criteria [International Myeloma Working Group, 2003 (22)], and the presence of >60% plasma cells in the bone marrow. A total of 18 healthy donors [10 males, 8 females; median age, 50 years (range, 45-68 years)] were recruited as a control group. The study was approved by the Institutional Review Board of Affiliated Union Hospital of Fujian Medical University, and informed consent was obtained from all subjects prior to sample collection.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Mononuclear cells (MNCs) were isolated from patient bone marrow samples using Ficoll-Hypaque density gradient centrifugation (Inno-Train Diagnostik GmbH, Kronberg im Taunus, Germany). Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). To determine miR expression, total RNA (1 μl/sample) was reverse-transcribed using miR-specific stem-loop RT primers, reverse transcriptase, RT buffer, dNTPs and an RNase inhibitor, according to the manufacturer's protocol (TaqMan® MicroRNA Reverse Transcription kit; Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using an Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.). The 20-μl reaction mixture contained the corresponding complementary DNA (2 μl), miRNA-specific TaqMan primers (1 μl), TaqMan Universal PCR Master mix (10 μl; Applied Biosystems; Thermo Fisher Scientific, Inc.) and double-distilled H_2O (7 μl). The PCR cycling conditions were as follows: Denaturation at 95˚C for 10 min, followed by 50 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 1 min. RNU6B served as an endogenous housekeeping control for data normalization of miR levels. mRNA was reverse-transcribed in RT reactions containing 500 ng total RNA extracted from samples, 2 μl 5X PrimeScript™ Buffer (Takara Bio, Inc., Otsu, Japan), 0.5 μl 1X PrimeScript™ RT Enzyme Mix I (Takara Bio, Inc.) and 0.5 μl oligo (dT) primer. The 10-μl reactions were incubated for 42 min at 37˚C, followed by 30 sec at 85˚C and subsequent exposure to 4˚C. The endogenous mRNA levels of Bmi-1 were detected using the SYBR® Green PCR Master mix kit (Takara Bio, Inc.), according to the manufacturer's protocol. The PCR cycling conditions were as follows: An initial denaturation step at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. β-actin served as an internal control. The following primers (Invitrogen; Thermo Fisher Scientific, Inc.) were used: Forward, 5'-AAATCAGGGGGTTGAAAAATCT-3' and reverse, 5'-GCTAACCACCAATCTTCCTTTG-3' for Bmi-1; and forward, 5'-TTGTTACAGGAAGTCCCTTGCC-3' and reverse, 5'-ATGCTATCACCTCCCCTGTGTG-3' for β-actin. The comparative quantification cycle (Cq) method was used to measure the relative changes in expression; $2^{-\Delta\Delta Cq}$ represents the fold-change in expression (23).

Cell culture. HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). U266 and RPMI8226 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin.

Lentivirus production and infection. The pri-miR-203 sequence was amplified and cloned into the pWPXL lentiviral vector (donated by Dr Didier Trono, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). The primers were as follows: Forward, 5'-GAATTCCGTCTAAGGCGTCCG GTA-3' and reverse, 5'-GCGGCCGCGTTCCCACAGCAC AGC-3'. The pWPXL vectors were transfected into HEK-293T cells with the packaging plasmid psPAX2 and the VSV-G envelope plasmid pMD2.G (donated by Dr Didier Trono) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cell supernatants were collected at 48 h post-transfection and passed through a 0.22-mm filter. The virus particles were harvested 48 h following transfection. The titer of purified virus was 2.0x108 IU/ml. Cells ($1x10^5$) were infected with $1x10^6$ recombinant lentivirus-transducing units and 6 μg/ml Polybrene (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany).

Cell proliferation. Cell proliferation was measured using the Cell Counting kit (CCK)-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were seeded into 96-well plates ($4x10^3$/well), and 10 μl CCK-8 solution was added to 90 μl culture medium. Cells were subsequently incubated for 2 h at 37˚C and the optical density was measured at 450 nm and 650 nm. Three independent experiments were performed.

Cell cycle and apoptosis determination by flow cytometry. For cell cycle analysis, cells were collected and fixed in ice-cold 70% ethanol overnight. The fixed cells were washed with phosphate-buffered saline (PBS) and stained with freshly-prepared PBS containing 25 μg/ml propidium iodide (Sigma), 10 μg/ml RNaseA, 0.05 mM ethylene diamine and 0.2% Triton X-100 tetra-acetic acid for 30 min in the absence of light. For each sample, at least 20,000 cells were acquired on an EPICS Altra flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and analyzed using Multicycle AV software version 5.0 (Phoenix Flow Systems, San Diego, CA, USA).

For apoptosis detection, cells ($2x10^5$) were collected and stained with an Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (AAD) Apoptosis Detection kit (Sigma-Aldrich; Merck Millipore) according to the manufacturer's protocol. Cells were acquired and analyzed as above.

Oligonucleotide transfection. The miR-203 mimic and negative control were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of the RNA duplex control was 5'-UUCUCCGAACGUGUCACGU-3'. Oligonucleotide transfection was performed with Lipofectamine 2000.

Luciferase reporter constructs and luciferase assay. The wild-type and mutant 3'UTRs of Bmi-1 were cloned downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pCDNA3.0 vector (Thermo Fisher Scientific, Inc.). The primers used were as follows: Forward, 5'-AGTGGACGTTCACCGAGTT-3' and reverse, 5'-TCCCAA

AAGCGCATTTATT-3' for wild-type; and forward, 5'-CTA GAATGAAGATGTCCCCCATCTTATACCCCTAAC-3' and reverse, 5'-GATGGGCGGACATCTTCTTTCTAGCAGGGA GACACTG-3' for mutant. For the luciferase assay, 10,000 U266 cells were cultured in 24-well plates and cotransfected with 20 pmol RNA (negative control or miR-203 mimic), 200 ng luciferase reporter construct and 20 ng pRL-CMV renilla luciferase reporter construct. Following a 48-h incubation, luciferase activity was measured using the Dual-Luciferase® Reporter assay system (Promega Corporation, Madison, WI, USA).

Western blotting. Cells were washed with cold PBS and lysed in culture dishes using PhosphoSafe™ extraction reagent (Merck Millipore) containing 1% protease inhibitor cocktail (EDTA-free; Thermo Fisher Scientific, Inc.). Protein concentrations were then determined using Bio-Rad detergent-compatible protein assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (50 μg) were loaded onto 12% SDS-PAGE gels, electrophoresed and transferred onto polyvinylidene difluoride membranes (0.22 μm; Merck Millipore). Membranes were blocked with 5% nonfat milk and incubated with rabbit anti-Bmi-1 monoclonal antibody (1:1,000; catalog no. 2830S; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit polyclonal anti-β-actin (1:1,000; catalog no. ab119716; Abcam, Cambridge, MA, USA) for 1.5 h at room temperature. Following washing, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (1:1,000; catalog no. bs-0295M; Bioss Inc., Woburn, MA, USA). Protein bands were visualized using an Enhanced Chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error. The data were subjected to two-tailed Student's *t*-test. Linear regression analysis was used to investigate the relationship between the expression of miR-203 and Bmi-1. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-203 downregulates Bmi-1 expression by directly targeting the Bmi-1 3'UTR. Bmi-1 is upregulated significantly in various malignancies including MM, and may be post-transcriptionally regulated by miRNAs. Therefore, Bmi-1 may be regulated by miRNAs in MM. The online miRNA target-prediction tools, TargetScan (www.targetscan.org/vert_71/), PicTar (pictar.mdc-berlin.de/) and miRanda (www.microrna.org/microrna/home.do), were used to predict Bmi-1-targeting miRNAs. The number of potential Bmi-1-targeting miRNAs was 16, 12 and 82 for TargetScan, PicTar and miRanda, respectively. Altogether, a pool of 86 miRNAs that may potentially target Bmi-1 was selected. Of these miRNAs, miR-203 has been reported as a tumor suppressor miRNA inhibiting cellular proliferation by targeting CREB1 mRNA in MM (20). Therefore, the present study focused on miR-203. To determine whether miR-203 exerts its function by downregulating the expression of Bmi-1 through direct binding to its 3'UTR, full-length fragments of Bmi-1 mRNA 3'UTR (wild-type or mutant) were constructed and inserted immediately downstream of the luciferase reporter gene. For the luciferase assays, the miR-203 mimic was cotransfected with the luciferase 3'UTR constructs into U266 cells and RPMI8226 cells. The results revealed that miR-203 decreased the relative luciferase activity when transfected with wild-type, but not mutant, Bmi-1 3'UTR ($P=0.022$ and $P=0.016$ for U266 and RPMI8226 cells, respectively; Fig. 1A). In accordance with these results, a marked decrease was observed in endogenous Bmi-1 protein (Fig. 1B) and mRNA ($P=0.018$ and $P=0.024$ for U266 and RPMI8226 cells, respectively; Fig. 1C) expression levels in U266 cells and RPMI8226 cells following infection with miR-203-expressing lentivirus. Taken together, these results suggested that miR-203 may downregulate Bmi-1 expression post-transcriptionally by directly targeting its 3'UTR.

Enforced expression of miR-203 inhibits MM cell growth and cell cycle transition. To determine the functional role of miR-203 in MM, a lentivirus vector harboring miR-203 was constructed and used to establish two stable MM cell lines, U266-203 and RPMI8226-203. Analysis of proliferation (Fig. 2A and B) revealed that ectopic expression of miR-203 resulted in a significant decrease in the growth of U266-203 (day 3, $P=0.0215$; day 5, $P=0.0129$) and RPMI8226-203 (day 3, $P=0.0198$; day 5, $P=0.0095$) cells. As miR-203 inhibits MM cell proliferation, the effect of miR-203 on MM cell cycle progression was investigated. The cell cycle distribution demonstrated that the proportion of cells in G_1 phase was significantly increased in U266-203 ($P=0.0158$) and RPMI8226-203 ($P=0.0142$) cells compared with control vector, whereas the cell population in S phase was significantly reduced in U266-203 ($P=0.0307$) and RPMI8226-203 ($P=0.0134$) cells (Fig. 2C and D). Therefore, the growth-inhibiting effect of miR-203 on MM cells may be due to the arrest of cell cycle progression at the G_1/S transition.

Ectopic expression of miR-203 promotes MM cell apoptosis. Annexin V-PE/7-AAD staining was performed to evaluate the effect of miR-203 overexpression on MM cell apoptosis. The percentage of apoptotic U266-control and U266-203 cells was 1.5±0.008 and 36±2.04% ($P<0.001$), respectively (Fig. 3A); the percentage of apoptotic RPMI8226-control and RPMI8226-203 cells was 13.66±0.98 and 30.06±1.65% ($P=0.014$), respectively (Fig. 3B). Therefore, ectopic expression of miR-203 may promote MM cell apoptosis by targeting Bmi-1.

miR-203 is downregulated in MM and negatively correlates with Bmi-1 expression. To determine miR-203 expression levels in bone marrow MNCs from newly diagnosed MM patients and healthy donors, RT-qPCR was performed. miR-203 was significantly downregulated in MM patients compared with healthy controls ($P<0.001$; Fig. 4A). Bmi-1 was relatively highly expressed in MM and negatively correlated with miR-203 expression ($P=0.0128$; Fig. 4B). Furthermore, miR-203 expression levels were examined in certain patients following treatment remission and relapse. miR-203 expression increased following remission and declined again

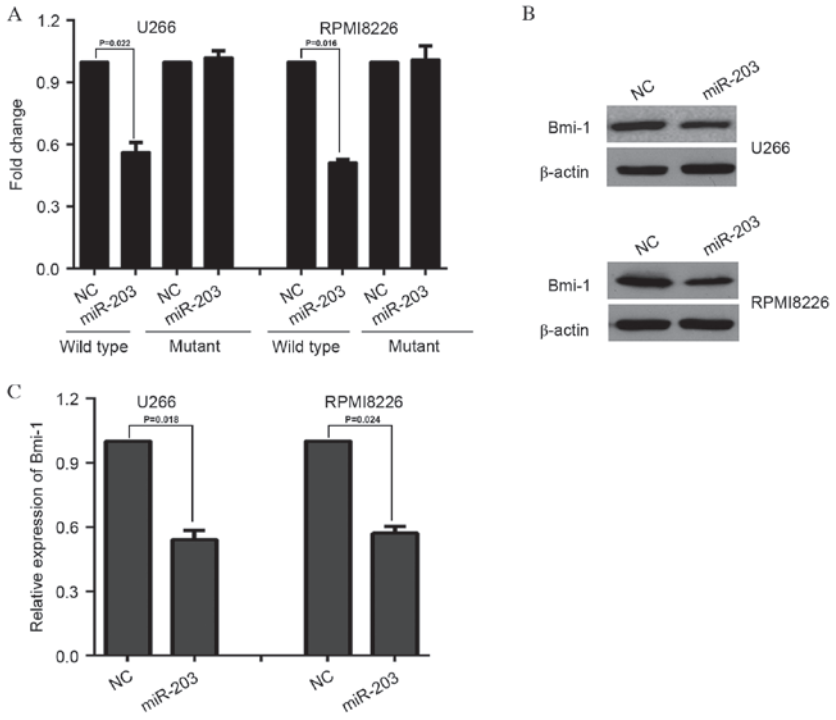

Figure 1. miR-203 downregulates Bmi-1 expression by directly targeting the Bmi-1 3'UTR. (A) Luciferase activity assays were performed following cotransfection of wild-type or mutant Bmi-1 3'UTRs with an miR-203 mimic or negative control in U266 and RPMI8226 cells. The luciferase activity of each sample was normalized to Renilla luciferase activity. miR-203 decreased the relative luciferase activity when transfected with wild-type, but not mutant, Bmi-1 3'UTR. Bmi-1 (B) protein and (C) mRNA expression levels were determined by western blot and reverse transcription-quantitative polymerase chain reaction analysis, respectively, following transfection of U266 and RPMI8226 cells with miR-203 mimics or negative control. miR-203 decreased Bmi-1 protein and mRNA expression levels compared with the negative control. β-actin served as an internal control. Data are expressed as the mean ± standard error. Statistical analysis was performed using the Student's *t*-test. miR, microRNA; Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site-1; UTR, untranslated region; NC, negative control.

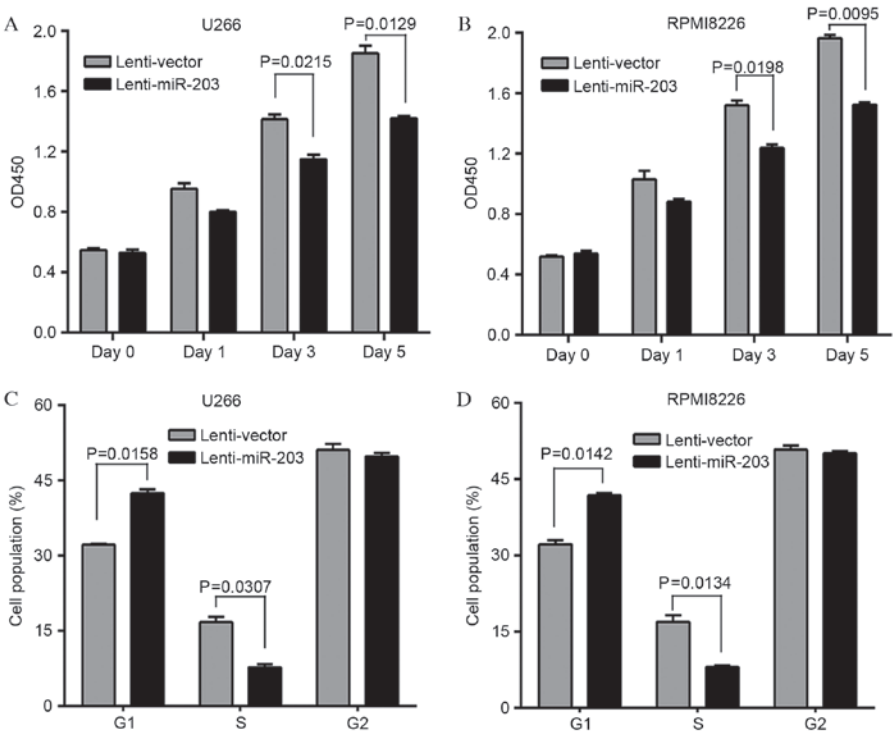

Figure 2. Enforced expression of miR-203 inhibits MM cell growth and cell cycle transition. Proliferation of (A) U266 and (B) RPMI8226 cells was measured following infection with miR-203-expressing or control lentivirus. miR-203-expressing cells had decreased growth compared with controls. Cell cycle analysis of (C) U266 and (D) RPMI8226 cells was performed by flow cytometry following infection with miR-203-expressing or control lentivirus. miR-203 inhibited G_1/S progression. Data are expressed as the mean ± standard error of triplicate experiments. Statistical analysis was performed using the Student's *t*-test. miR, microRNA.

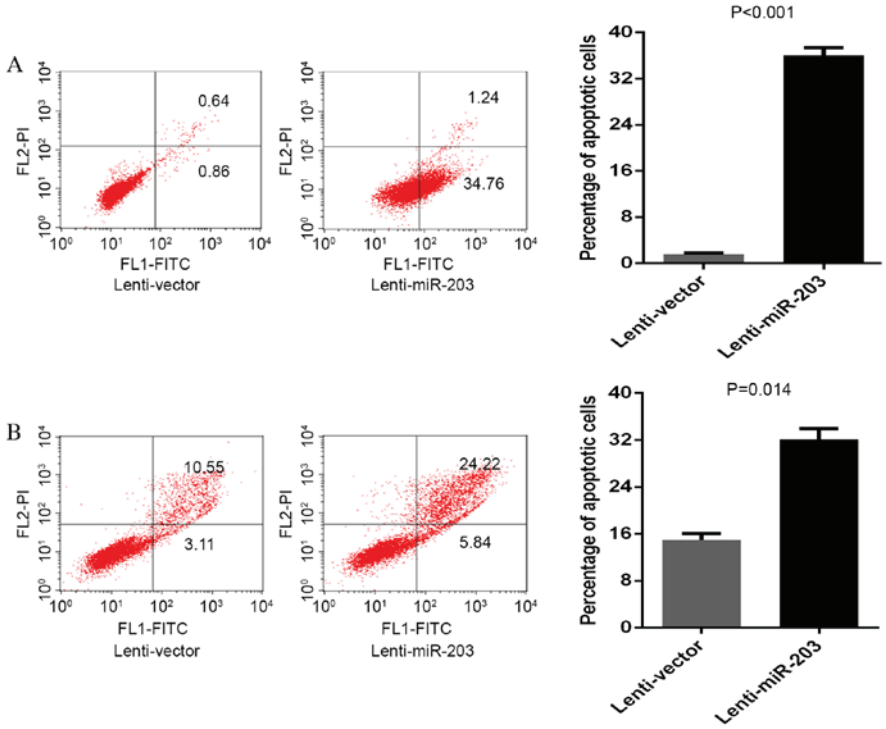

Figure 3. Ectopic expression of miR-203 promotes cell apoptosis in MM. Apoptosis of (A) U266 and (B) RPMI8226 cells was measured following infection with miR-203-expressing or control lentivirus, using Annexin V-PE/7-AAD staining. The percentage of apoptotic cells was increased in miR-203-expressing cells compared with control. The values in the lower-right (Annexin V^-/7-AAD^+) and upper-right (Annexin V^+/7-AAD^+) quadrants represent the percentage of early- and late-apoptotic cells, respectively. miR, microRNA; PE, phycoerythrin; 7-AAD, 7-aminoactinomycin D.

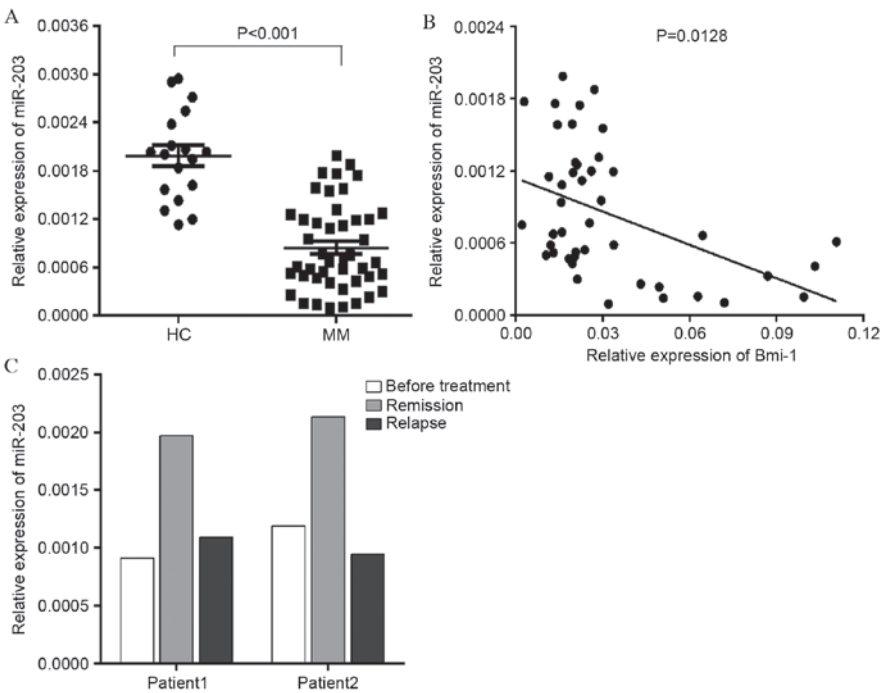

Figure 4. Expression levels of miR-203 and Bmi-1 in MM patients. (A) miR-203 expression levels were decreased in MM patients compared with healthy controls. (B) Bmi-1 expression in MM negatively correlated with miR-203 expression. (C) miR-203 expression levels in MM patients increased following remission and decreased again following relapse. miR, microRNA; Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site-1; HC, healthy control; MM, multiple myeloma.

following relapse (Fig. 4C). Taken together, these results demonstrated that the expression levels of miR-203 were downregulated in MM patients, and may be associated with the current disease state.

Discussion

Bmi-1 was originally described as interacting with c-Myc to initiate lymphoma in mice (1). Evidence has demonstrated that Bmi-1 is critical for numerous physiological and pathological processes, including hematopoiesis (3), senescence (4), axial patterning (2), regulation of proliferation and maintenance of cancer stem cell self-renewal (5). In addition, evidence has suggested that Bmi-1 is upregulated in various malignancies, including colorectal cancer (6), non-small cell lung cancer (8), pancreatic cancer (24), breast cancer (25), prostate cancer (26) and leukemia (7). Bmi-1 is overexpressed in MM and may regulate cell proliferation and carcinogenesis *in vitro* and *in vivo*. Our previous study demonstrated that silencing of Bmi-1 sensitizes MM cells to bortezomib (10). However, the reasons behind the upregulation of Bmi-1 in MM remain unclear.

miRNAs are post-transcriptional regulators of downstream gene expression, and may function as oncogenes or tumor suppressor genes during tumor development and progression (27). Previous studies have suggested that Bmi-1 may be post-transcriptionally regulated by miRNAs (11-14); however, whether miRNAs target Bmi-1 in MM remains unclear. In the present study, three miRNA target-prediction tools, TargetScan, PicTar and miRanda, were used to predict all the miRNAs that may target Bmi-1. A total of 86 miRNAs were identified that may target Bmi-1. Of these miRNAs, miR-203 was selected for further investigation. Previous studies have reported that miR-203 is downregulated in MM and CML due to epigenetic silencing, and that CREB1 is a novel target of miR-203 in MM (19,20). However, computational analysis indicates that one miRNA may regulate multiple mRNAs. Therefore, more mRNAs than CREB1 may be regulated by miR-203 in MM. The present study identified Bmi-1 as a direct downstream target of miR-203 in MM cells. A potential miR-203 target site was identified in the Bmi-1 3'UTR. Following cotransfection with wild-type 3'UTR, miR-203 decreased the relative luciferase activity. However, when the potential target site was mutated, luciferase activity was unaffected. In accordance with these results, endogenous Bmi-1 protein and mRNA expression levels were demonstrated to be downregulated by miR-203 in MM cells.

If miR-203 functions as a tumor suppressor in MM, restoration of miR-203 expression in MM cells may inhibit proliferation and/or promote apoptosis. The present study demonstrated that ectopic expression of miR-203 in MM cells led to a significant inhibition of proliferation and the arrest of cell cycle progression at the G_1/S transition. In addition, the present study suggested that miR-203 re-expression may promote MM cell apoptosis by targeting Bmi-1. Taken together, these results confirm miR-203 as a tumor suppressor in MM. Furthermore, the expression levels of miR-203 and Bmi-1 in MM patient and healthy control bone marrow samples were detected. miR-203 was significantly downregulated in MM; Bmi-1 was relatively abundant in MM and negatively correlated with miR-203 expression. In certain clinical circumstances, miRNA profiling may be superior to mRNA profiling to classify tumor subtypes and predict prognosis. The present study demonstrated that miR-203 expression may indicate current disease status.

In conclusion, the results of the present study demonstrated, to the best of our knowledge for the first time, that miR-203 may target Bmi-1 in MM, inhibit MM cell growth and regulate G_1/S transition. These results suggested that miR-203 has tumor suppressor properties and therefore potential therapeutic application in MM.

Acknowledgements

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