

MicroRNA-218 Inhibits Cell Cycle Progression and Promotes Apoptosis in Colon Cancer by Downregulating BMI1 Polycomb Ring Finger Oncogene

Xinqi He,^{1,2*} Yujuan Dong,^{1,3*} Chung Wah Wu,¹ Zengren Zhao,² Simon S M Ng,³ Francis K L Chan,¹ Joseph J Y Sung,¹ and Jun Yu¹

¹Institute of Digestive Disease and Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, Shenzhen Research Institute, The Chinese University of Hong Kong, Hong Kong; ²Department of Surgery, First Affiliated Hospital, Hebei Medical University, Shijiazhuang, China; and ³Department of Surgery, The Chinese University of Hong Kong, Hong Kong

Deregulated miRNAs participate in colorectal carcinogenesis. In this study, miR-218 was found to be downregulated in human colorectal cancer (CRC) by miRNA profile assay. miR-218 was silenced or downregulated in all five colon cancer cells (Caco2, HT29, SW620, HCT116 and LoVo) relative to normal colon tissues. miR-218 expression was significantly lower in 46 CRC tumor tissues compared with their adjacent normal tissues ($P < 0.001$). Potential target genes of miR-218 were predicted and BMI1 polycomb ring finger oncogene (BMI-1), a polycomb ring finger oncogene, was identified as one of the potential targets. Upregulation of BMI-1 was detected in CRC tumors compared with adjacent normal tissues ($P < 0.001$) and in all five colon cancer cell lines. Transfection of miR-218 in colon cancer cell lines (HCT116, HT29) significantly reduced luciferase activity of the wild-type construct of *BMI-1* 3' untranslated region (3'UTR) ($P < 0.001$), whereas this effect was not seen in the construct with mutant *BMI-1* 3'UTR, indicating a direct and specific interaction of miR-218 with BMI-1. Ectopic expression of miR-218 in HCT116 and HT29 cells suppressed BMI-1 mRNA and protein expression. In addition, miR-218 suppressed protein expression of BMI-1 downstream targets of cyclin-dependent kinase 4, a cell cycle regulator, while upregulating protein expression of p53. We further revealed that miR-218 induced apoptosis ($P < 0.01$), inhibited cell proliferation ($P < 0.05$) and promoted cell cycle arrest in the G2 phase ($P < 0.01$). In conclusion, miR-218 plays a pivotal role in CRC development through inhibiting cell proliferation and cycle progression and promoting apoptosis by downregulating BMI-1.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2012.00304

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and the third leading cause of cancer-related death in the world, with an estimated incidence of 1 million new cases and a mortality of >600,000 deaths annually (1,2). Recent progress in diagnosis and therapy has

helped to save the lives of many patients at early stages of this malignancy, but the prognosis for patients with advanced disease or metastasis is still very poor. Therefore, further investigation into the molecular pathogenesis of CRC and the consequential development of novel targeted therapeutics are needed. A series

of studies have revealed that microRNAs (miRNAs) can regulate the expression of a variety of genes pivotal for tumor development and highlight a novel mechanism participating in CRC pathogenesis (3–5).

miRNAs are 21- to 23-nucleotide RNAs that negatively regulate gene expression by binding to the 3' untranslated regions (3'UTRs) of target transcripts, leading to mRNA degradation or inhibiting translation into protein. miRNAs are reported to play an important role in the pathogenesis of human cancers with disordered genome function (6,7). This result also applies to CRC; a subset of miRNAs was found to be aberrantly expressed in CRC, and most of the miRNAs are related to cell proliferation, apoptosis and tumor metastasis (4,8). By using miRNA arrays to com-

*XH and YD are co-first authors.

Address correspondence to Jun Yu, Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong. Phone: +852-37636099; Fax: +852-21445330; E-mail: junyu@cuhk.edu.hk; or Zengren Zhao, Department of Surgery, First Affiliated Hospital, Hebei Medical University, Shijiazhuang, China. Phone: +86-311-85917006; Fax: +86-311-85917007; E-mail: zzi-doctor@163.com. Submitted August 26, 2012; Accepted for publication December 11, 2012; Epub (www.molmed.org) ahead of print December 12, 2012.

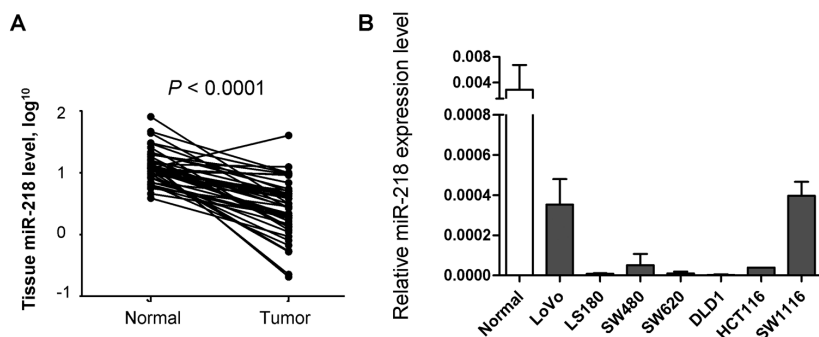


Figure 1. miR-218 is commonly downregulated in primary colorectal cancer tissues and colon cancer cell lines. (A) Levels of miR-218 in 46 primary colorectal tumors compared with their adjacent normal tissues. The miR-218 level is normalized to RNU6B, and the P value indicated a significant difference in miR-218 level between paired samples determined by the Wilcoxon matched pair test. (B) The expression level of miR-218 in five colon cancer cell lines and normal colon tissues ($n = 5$) by quantitative RT-PCR with RNU6B as an internal control.

pare the miRNA profiles in the CRC tissue samples and normal controls, we recently identified a promising candidate that is significantly downregulated in CRCs (miR-218). The identification and characterization of the role of novel miRNA in CRC may provide new insight into understanding the molecular mechanisms of disease development and is therefore crucial for the development of new therapy.

In the present study, we validated the downregulation of miR-218 by examining 48 paired CRC tumor and adjacent nontumor tissue samples. A series of *in vitro* and *in vivo* studies were then conducted to investigate the mechanisms and impact of miR-218 in colon cancer. We systematically discovered miR-218-mediated targets *in silico* by the algorithms TargetScan and MiRanda and experimentally validated that the repressions of BMI1 polycomb ring finger oncogene (BMI-1) was directly regulated by miR-218. We further demonstrated that ectopic expression of miR-218 in colon cancer cells led to reduced cell proliferation and induced apoptosis. Additionally, we demonstrate that miR-218 induces cell cycle arrest in the G2 phase of colon cancer cells by suppressing cyclin-dependent kinase 4 (CDK4) and up-regulating p53, two downstream targets of BMI-1.

MATERIALS AND METHODS

Cell Culture

Colon cancer cell lines HCT116, HT29, SW620 and LoVo were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 and HT29 were cultured in McCoy's 5A medium (Invitrogen; Life Technologies, Carlsbad, CA, USA), and SW620 and LoVo were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). All the cells were cultured in a humidified 37°C incubator supplemented with 5% CO₂.

Human Samples

Paired resected surgical specimens from primary tumor and adjacent nontumor sites were obtained from CRC patients who underwent surgery at the Prince of Wales Hospital, Hong Kong, according to a standard protocol, before any therapeutic intervention. The adjacent macroscopically nontumor mucosa at least 6 cm distant from the tumor was taken. The specimens were snap-frozen in liquid nitrogen and stored at -80°C for molecular analyses. The remaining tissue specimens were fixed in 10% formalin and embedded in paraffin for routine histological examination. All subjects provided informed consent before specimen collection. The study protocol was

approved by the Ethics Committee of The Chinese University of Hong Kong.

Microdissection and RNA Extraction from Paraffin-Embedded Tissues

Five paraffin-embedded normal colon tissues were cut into 10- μ m thickness sections by using a microtome. The muscularis mucosae in normal tissues were then excluded by laser-captured microdissection under a microscope. Total RNA was extracted from the five microdissected normal colon tissues by using a RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA).

miRNA Extraction

Total RNA was extracted from tissue samples (10–30 mg) by using Trizol reagent (Invitrogen; Life Technologies). Total RNA was eluted in 50 μ L nuclease-free water. RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA).

miRNA Array

Reverse transcription for miRNA microarray was performed by using Megaplex Primer pools (Human Pools A v2.1 and B v3.0; Applied Biosystems; Life Technologies). The cDNA products of miRNAs in the two pairs of tumor tissues and adjacent nontumor tissues of two CRC patients were used to perform miRNA array. The miRNA profile of 754 human miRNAs was obtained from each sample by using a TaqMan Human MicroRNA Array Set v2.0 (Applied Biosystems; Life Technologies). Briefly, 6 μ L Megaplex RT product from each sample was added to 444 μ L nuclease-free water and 450 μ L TaqMan Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems; Life Technologies). The reaction mix (100 μ L) was loaded into each of the eight fill reservoirs of the array. Real-time quantitative polymerase chain reaction (PCR) was performed by using an Applied Biosystems 7900HT Real-Time PCR System (Life Technologies). Results were analyzed by the SDS RQ Manager 1.2 software (Applied Biosystems; Life Technologies).

Real-Time Quantitative PCR for miRNA and mRNA Expression Analyses

Quantitative real-time PCR (qRT-PCR) of individual miRNA was performed by using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems; Life Technologies) and TaqMan Human miRNA Assay kit (Applied Biosystems; Life Technologies). The comparative cycle threshold (Ct) method was used to calculate the relative abundance of miRNA compared with RNA U6 small nuclear 2 (RNU6B) expression (fold difference relative to RNU6B).

For *BMI-1* mRNA quantification, RNA was reverse-transcribed with a random primer by using an ABI[®] Reverse Transcription Kit (Applied Biosystems; Life Technologies). mRNA levels were determined with qRT-PCR by using the SYBR Green master mixture on an HT7500 System (Applied Biosystems; Life Technologies) with the housekeeping gene *GAPDH* as an internal control. *BMI-1* primers were as follows: forward 5'-GTGCT TTGTG GAGGG TACTT CAT-3'; reverse 5'-TTGGA CATCA CAAAT AGGAC AATAC TT-3'.

Western Blot

Total protein was extracted and protein concentration was measured by the Bradford DC protein assay (Bio-Rad, Hercules, CA, USA). Then, 20–40 μ g protein from each sample were separated on 12% Bis-Tris polyacrylamide gel through electrophoresis and blotted onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Blots were immunostained with primary antibody at 4°C overnight and secondary antibody at room temperature for 1 h. Proteins were visualized by using ECL Plus Western Blotting Detection Reagents (RPN2132; GE Healthcare).

Colony Formation Assay

Cells (1×10^5 /well) were plated in a 24-well plate and transfected with pre-miR-218 or pre-miR control (control) at 30 nmol/L by using lipofectamine 2000 (Invitrogen; Life Technologies). After 48 h, the cells were collected and seeded

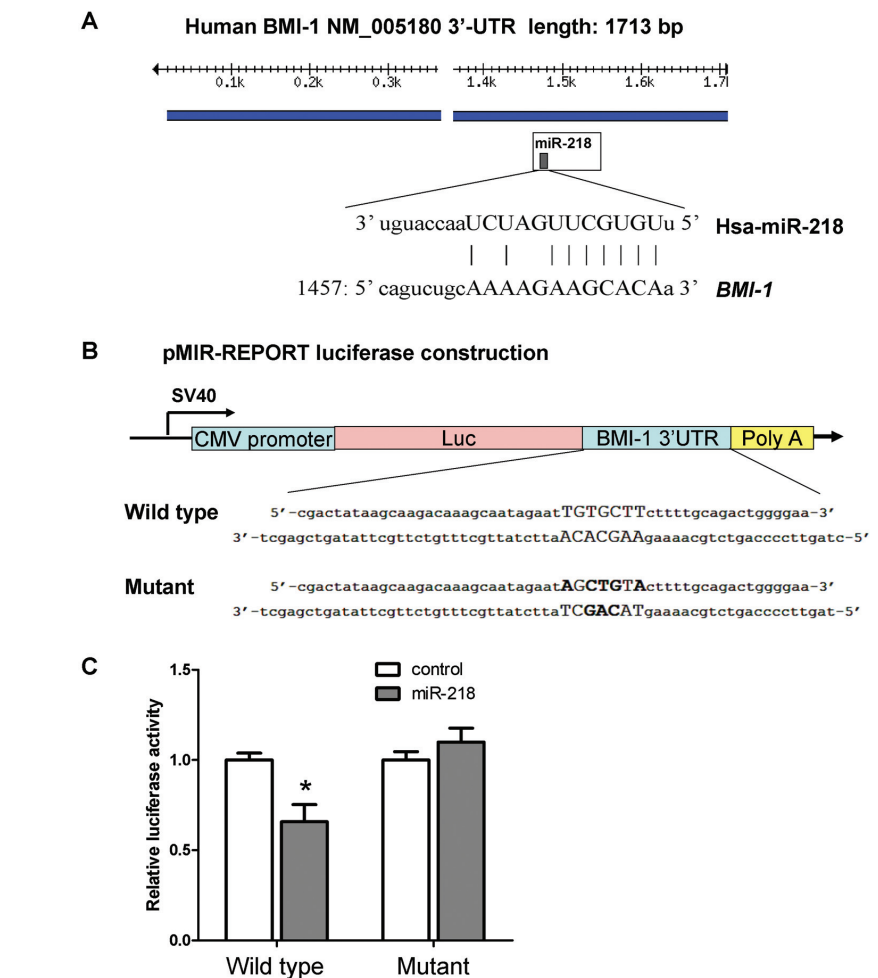


Figure 2. *BMI-1* is a direct target of miR-218. (A) Conservation of *BMI-1* 3'UTR binding site for miR-218 among different species. (B) The conserved miR-218 binding sequence or its mutated form was inserted into the C-terminal of the luciferase gene to generate pMIR-*BMI-1*-3'UTR or pMIR-*BMI-1*-mut-3'UTR, respectively. (C) miR-218 targets the wild-type but not the mutant 3'UTR of *BMI-1*. miR-218 was cotransfected with pMIR-REPORT (the empty vector) or reporters constructed containing either wild-type or mutant *BMI-1* 3'UTR into HCT116 cells. pRL-CMV was used as an internal control reporter ($n = 3$, mean \pm SD). The luciferase activity assay was repeated three times in triplicate. * $P < 0.05$.

(500–1,000/well) in a fresh 12-well plate for 10 d. The cells were stained with hematoxylin solution and the individual colony (>50 cells) was counted.

Apoptosis Assay

Cells (1×10^5 /well) were collected 48 h after transfection with pre-miR-218 or control and were stained with Annexin V (allophycocyanin [APC] conjugated) and 7-aminoactinomycin D (7-AAD) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Bel-

gium). Apoptosis was assessed by flow cytometry (BD FACSCalibur).

Vector Construction and Dual-Luciferase Reporter Assay

The potential miR-218 binding site in the *BMI-1* 3'UTR was predicted at position 1,470–1,476 from the transcriptional start site of *BMI-1* (NM_005180) by TargetScan (www.targetscan.org) and miRanda (www.microRNA.org). Sequence of the 50-bp segment with the wild-type or mutant seed region was synthesized and

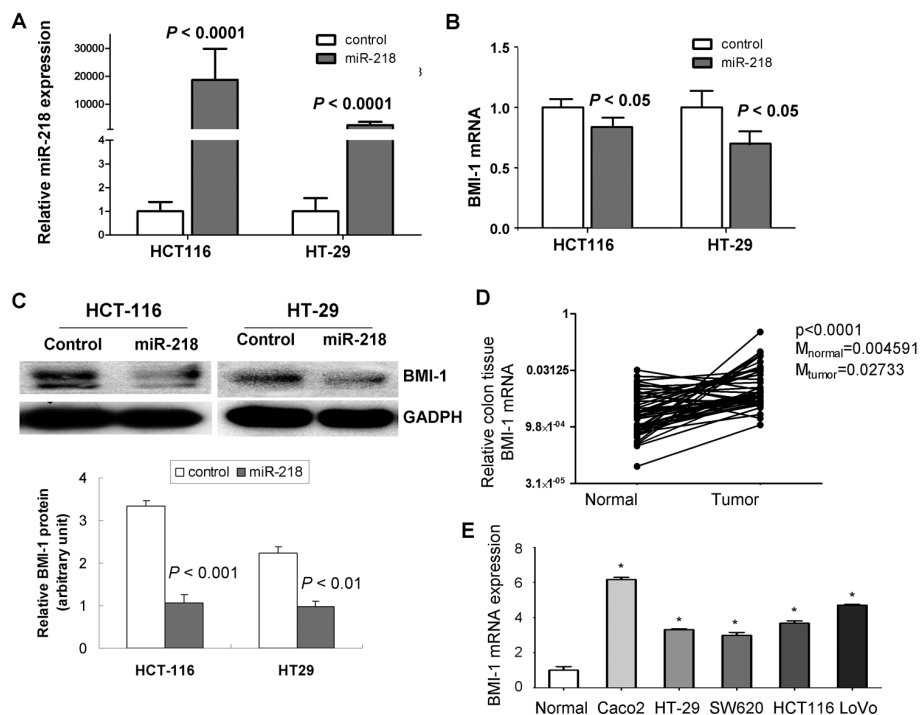


Figure 3. Transfection of miR-218 decreases BMI-1. (A) Overexpression of miR-218 was demonstrated in HCT116 and HT29 cell lines by quantitative RT-PCR. (B) Ectopic expression of miR-218 downregulated the BMI-1 mRNA level in HCT116 and HT29 cells, as determined by quantitative real-time PCR. (C) miR-218 decreased the BMI-1 protein level in HCT116 and HT29 cells, as determined by Western blot. The BMI-1 protein band intensities were quantified and normalized to GAPDH intensities ($n = 3$, mean \pm SD). (D) Levels of BMI-1 mRNA is upregulated in primary colorectal tumors compared with their adjacent normal tissues ($n = 46$). (E) BMI-1 mRNA is upregulated in colon cancer cell lines compared with five normal colon tissues.

cloned into the pMIR-REPORT luciferase vector (Applied Biosystems; Life Technologies). The mutant *BMI-1* 3'UTR sequence was prepared by mutating five nucleotides in the seed region. The synthesized oligos were shown as follows: wild-type: 5'-cgact ataa caaga caag caata gaat GTGCT Tctt tgcag actgg gaa-3', 3'-tcgag ctgat atcg ttctg ttctg ttatc ttaAC ACGAA gaaa cgtct gacc ctga tc-5'; mutant type: 5'-cgact ataa caaga caag caata gaatA GCTGT Actt tgcag actgg gaa-3', 3'-tcgag ctgat atcg ttctg ttctg ttatc ttaTC GACAT gaaa cgtct gacc ctga t-5'.

Cells (1×10^5 /well) transiently transfected with pre-miR-218 or control (at 30 nmol/L final concentration) were seeded in 24-well plates. pMIR-REPORT vector (195 ng/well) and pRL-cytomegalovirus (CMV) vector (5 ng/well) were cotrans-

ected by using lipofectamine 2000 (Invitrogen; Life Technologies). Cells were harvested 48 h after transfection, and luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

In Vivo Tumorigenicity

HCT116 cells (2×10^6 in 0.15 mL phosphate-buffered saline) were subcutaneously implanted into the left flank of the female nude mice (4 wks old) at d 0. After 5 d of implantation, control or miR-218 was injected into the tumor, and the injection was repeated every 3 d, respectively ($n = 4$ mice per group). miRNAs were prepared by preincubating 1.2 nmol miRNA with 10 μ L Lipofectamine 2000 (Invitrogen; Life Technologies) for 15 min, and injections were

made in a final volume of 100 μ L in McCoy's 5A medium (Sigma-Aldrich). Tumor sizes were measured every 3 d starting from the first injection with vernier calipers, and the tumor volume (V) was calculated as $(L \times w \times w)/2$, with L indicating length and w indicating width. The mice were euthanized when tumor size reached 12 mm in diameter and the tumors were excised. The animal studies and the experimental protocol were approved by the Department of Health of the Government of the Hong Kong Special Administrative Region and by the Committee on the Use of Live Animals in Teaching and Research of The Chinese University of Hong Kong.

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD). Differences between two groups in luciferase reporter assay and comet assay colony formation assay were determined by a Student t test. Difference in cell growth curves and *in vivo* tumorigenicity were determined by repeated-measures analysis of variance. All analyses were performed by using SPSS for Windows 11.0.1 software. $P < 0.05$ was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

miR-218 Was Downregulated from microRNA Array and Validated in the Primary CRC Tissues

Four patients with primary CRC were screened by using the miR array to identify miRNAs differentially expressed in CRC (data not shown). We identified 117 miRNAs downregulated in both samples. Among the candidate downregulated miRNAs (Supplementary Table S1), miR-218 showed a 2.5- to 10-fold decrease in expression level in the tumor samples compared with their adjacent nontumor samples.

To further confirm that miR-218 expression levels were different between tumor and nontumor tissues, we exam-

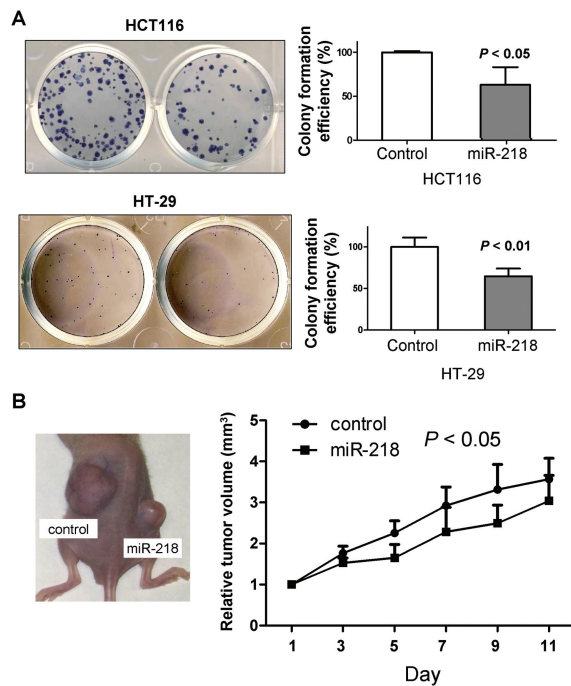


Figure 4. miR-218 inhibited colon cancer cell growth. (A) Ectopic expression of miR-218 in HCT116 and HT29 cells significantly inhibited colony formation. (B) miR-218 inhibited colon cancer cell growth *in vivo*. Tumor growth curve of miR-218 transfected cells in nude mice ($n = 4$, mean \pm SD).

ined 46 additional human CRC tumor samples. Consistent with the array result, there was a 3.6-fold decrease in the expression level of miR-218 in the 46 tumors than in their paired nontumor tissues (the mean of \log^{10} was 1.556 in the tumors and 0.4394 in the nontumor tissues; $P < 0.0001$) (Figure 1A). Among these paired samples, 97.8% (45/46) of the CRC samples showed lower miR-218 levels than in the adjacent normal tissue (see Figure 1A). We also examined miR-218 expression level in five colon cancer cell lines (HCT116, Caco2, SW620, HT29 and LoVo) and observed that miR-218 was downregulated in all five colon cancer cell lines relative to the microdissected normal primary colon cells from five normal tissues (Figure 1B). The association between miR-218 level and clinicopathological characteristics of colon cancer patients is analyzed. As shown in Supplementary Table S2, there is no correlation between miR-218 expression and clinicopathological features such as age, sex, tumor stage and recurrence.

miR-218 Inhibited the Expression of BMI-1 via Binding to Its 3'UTR

On the basis of two major prediction softwares, Targetscan (<http://www.targetscan.org>) and miRNA (<http://www.microrna.org>), potential binding sites of miR-218 in the 3'UTR of *BMI-1* were predicted (Figure 2A). To test the specific regulation through the predicted binding sites, we constructed a reporter vector consisting of the luciferase coding sequence followed by the 3'UTR of *BMI-1* (Luc-BMI-1 3'UTR) (Figure 2B). Mutant construct with the putative binding site (Luc-BMI-1-mut 3'UTR) was prepared (see Figure 2B). Cotransfection experiments showed that miR-218 decreased the luciferase activity of Luc-BMI-1 3'UTR for 34%, but this was not observed on Luc-BMI-1-mut 3'UTR (Figure 2C), indicating that *BMI-1* is a direct target of miR-218 through binding to 3'UTR of *BMI-1*.

To further confirm that miR-218 targets *BMI-1*, pre-miR-218 or control was transfected into HCT116 and HT29 cells; the transfection efficiency was determined

by real-time RT-PCR (Figure 3A). Transfection of miR-218 resulted in significant reduction of *BMI-1* mRNA (Figure 3B) and protein (Figure 3C) expression by real-time RT-PCR and Western blotting analysis, respectively. This result was concomitant with luciferase reporter activity results, inferring that miR-218 directly interacts with *BMI-1* 3'UTR to repress its translation.

BMI-1 Is Upregulated in Primary CRC Tissues

BMI-1 was reported to be a potential oncogene in human cancers (9). To corroborate the potential importance of *BMI-1* in primary CRCs, we compared the level of *BMI-1* expression in 46 paired tumors versus the surrounding normal tissues. The expression of the *BMI-1* mRNA accessed by real-time PCR was significantly increased in CRC tumors by about sixfold compared with the adjacent normal tissues ($P < 0.0001$; Figure 3D). *BMI-1* is overexpressed in 84.8% (39/46) of tumors compared with their normal counterparts (see Figure 3D). Moreover, the enhanced level of *BMI-1* mRNA was also detected in colon cancer cell lines compared with five normal colon tissues (Figure 3E).

BMI-1 Expression Correlates with miR-218 Expression in Primary CRC

To evaluate the correlation between *BMI-1* and miR-218 expression in primary CRCs, we compared the expression of *BMI-1* and miR-218 in 46 primary CRCs. Expression of *BMI-1* and miR-218 exhibited a significant inverse correlation calculated by Pearson correlation ($r = -0.823$, $P < 0.01$), further supporting the miR-218 target status of *BMI-1*.

miR-218 Inhibits Cell Growth of Colon Cancer Cell Lines

Previous studies demonstrated that *BMI-1* plays an important role in cell proliferation and acts as a potential oncogene in human cancers (9). We demonstrated that *BMI-1* is upregulated in CRC primary tissue as well as colon cancer cell lines. We thus supposed that miR-218, a direct target of *BMI-1*, might repress CRC

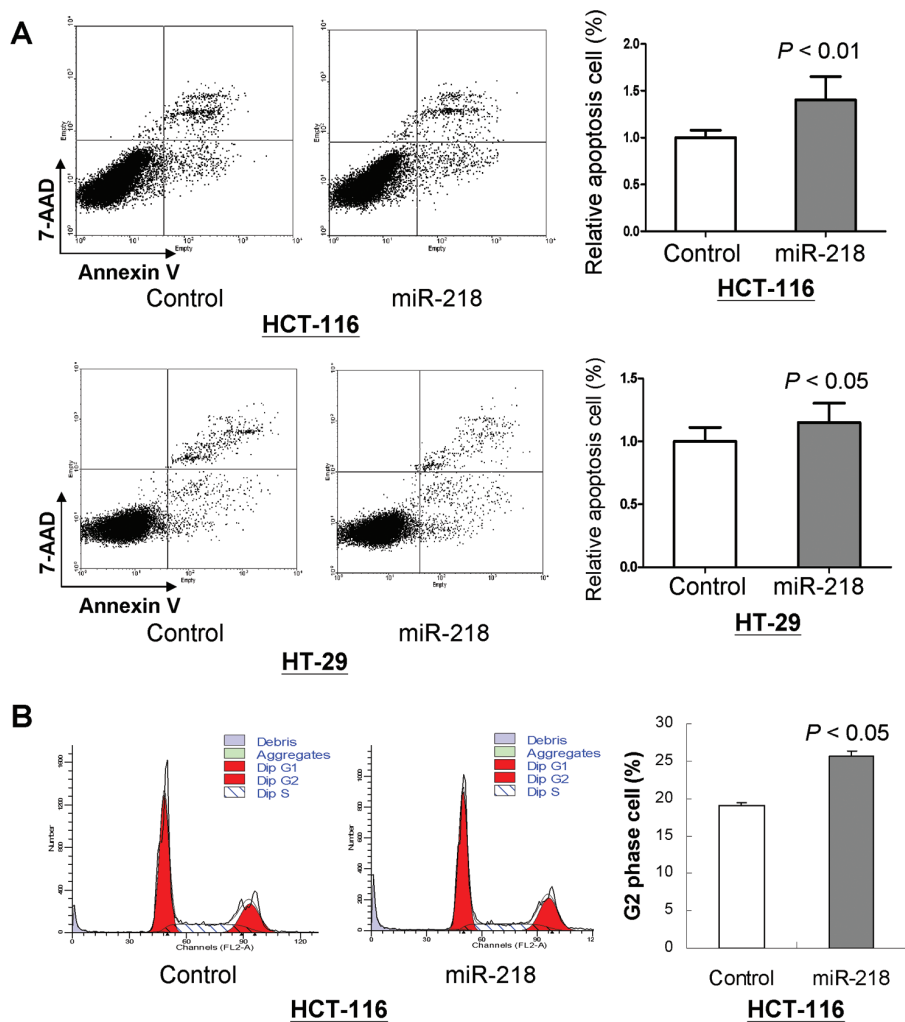


Figure 5. (A) miR-218 induced apoptosis of colon cancer cells. Cell apoptosis was examined by flow cytometry analysis of Annexin V APC and 7-AAD double staining. Region Q1 shows the necrotic cells, Q2 shows the late apoptotic cells, Q3 shows the live cells, and Q4 shows the early apoptotic cells. (B) miR-218 caused cell cycle arrest at G2 phase as demonstrated by flow cytometry.

growth through BMI-1 downregulation. To elucidate the functional significance of miR-218 in CRC, we examined the growth inhibitory effect through reexpression of miR-218 in two colon cancer cell lines (HCT116 and HT29). We observed that transduction with miR-218 led to a significant reduction of colony counts of the HCT116 ($P < 0.05$) and HT29 ($P < 0.01$) cells than transduction with miR-ctrl (Figure 4A). The inhibitory effect on the growth of colon cancer cells was further confirmed by tumorigenicity *in vivo*. The subcutaneous tumor growth

was lower in pre-miR-218-transfected HCT116 compared with the control-transfected HCT116 in nude mice (Figure 4B), inferring that miR-218 functions as a potential tumor suppressor in colorectal carcinogenesis.

miR-218 Induces Apoptosis in Colon Cancer Cell Lines

To identify the potential mechanism responsible for the observed effects of miR-218 on cell growth in CRC cells, we evaluated apoptosis rates in HCT116 and HT29 transduced with the miR-218 or

control vector. Apoptosis rates were evaluated via double staining of cells with Annexin V APC and 7-AAD, distinguishing between apoptotic cells and vital cells (Figure 5A). Transduction of miR-218 resulted in a significant increase in apoptotic cells compared with transduction of miR-ctrl both in HCT116 ($P < 0.01$) and HT29 ($P < 0.05$) cells (see Figure 5A).

miR-218 Induces Cell Cycle Arrest

Concomitant with this inhibition of cell proliferation by miR-218, there was a significant increase in the number of cells accumulating in the G2 phase ($P < 0.05$) (Figure 5B). Thus, miR-218 reduced cell proliferative capacity with a G2 cell cycle arrest.

miR-218 Upregulates p53 via BMI-1 in HCT116

BMI-1 was reported to regulate cell cycle and cell apoptosis through mediating CDK4 (4,10) and p53 (11). We considered that miR-218 might affect cells via the two pathways. Thus, we detected CDK4 and p53 protein level at 48 h after transfection. As expected, CDK4 was downregulated by miR-218 in both HCT116 and HT29. However, p53 was upregulated in HCT116 (wild-type p53), but not in HT29 (p53-defective) (Figure 6A). To evaluate if the increased p53 has biological activity, we transfected p53-luciferase, together with pre-miR-218 or control in HCT116, and found that the luciferase activity was raised by miR-218 restoration (Figure 6B).

DISCUSSION

In the present study, we identified that miR-218 is a predominant downregulated miRNA by using an miRNA profiling assay. By analyzing more CRC patients, we confirmed that miR-218 expression was significantly decreased in CRC tissues when compared with the adjacent nontumor tissues (Figure 1). Reduced miR-218 expression was also demonstrated in colon cancer cell lines compared with the normal colon tissues. Consistent with our results, downregulation of miR-218 was reported in other human cancers,

including gastric cancer (12), glioma (13), nasopharyngeal cancer (NPC) (14), lung cancer (15) and bladder cancer (16). Human miR-218 is encoded by two distinct genes (*miR-218-1* and *miR-218-2*), which are processed into an identical mature sequence (<http://www.mirbase.org>). miR-218-1 and miR-218-2 are embedded in the intronic regions of the *SLIT2* gene on chromosome 4p15.2 and *SLIT3* on chromosome 5q35.1, respectively. Frequent deletion of the 4p15.1-15.3 region occurs in CRC (17). Loss of heterozygosity in this region therefore may be associated with the downregulation of miR-218 in CRC patients. On the other hand, the host genes of the *SLIT* family are often inactivated in CRC through their promoter hypermethylation (18). We found that miR-218 expression was restored in CRC cell lines upon administration of the DNA methylation inhibitor 5-aza-2'-deoxycytidine (data not shown). In keeping with our finding, miR-218 was reported to be downregulated by promoter hypermethylation in nasopharyngeal carcinoma and oral squamous cell carcinoma (19,20). These data indicated that the CpG island hypermethylation may also potentially contribute to the silence of miR-218 in human CRC. The downregulation of miR-218 in CRC indicated that it may play a role in CRC tumorigenesis as a potential tumor suppressor.

To identify the miR-218 target gene, we applied combined *in silico* seed site analysis and luciferase reporter assay. We demonstrated that BMI-1 is a direct target of miR-218, with the evidence that overexpression of miR-218 led to reduced luciferase activity of *BMI-1* promoter and miR-218 downregulated BMI-1 expression (Figures 2, 3). BMI-1 was reported as an oncogene that regulates cell proliferation and transformation (21). BMI-1 is also crucial for self-renewal of stem cells and cancer initiation (22,23). We found that BMI-1 was overexpressed in CRC tumor tissues compared with adjacent normal tissues (Figure 3D). Enhanced BMI-1 expression was also observed in CRC cell lines. In keeping with our finding, other studies also reported

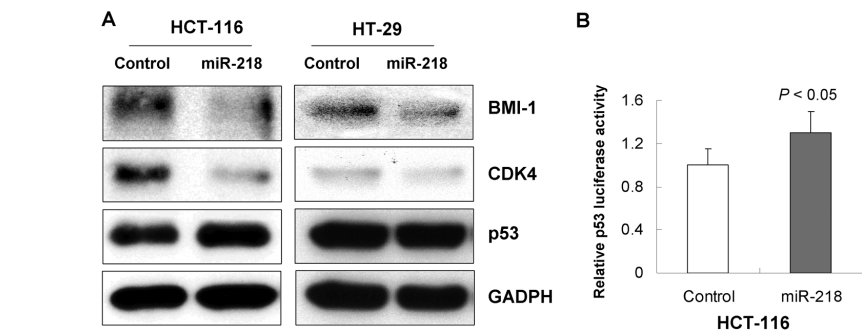


Figure 6. (A) Protein expression of BMI-1, CDK4 and p53 after miR-218 transfection in HCT116 and HT29 cells. (B) miR-218 induced luciferase reporter activity of p53.

that BMI-1 was overexpressed in CRC (24,25) and its overexpression was correlated with the malignant grades in precancerous lesions (25) and a shorter survival of CRC patients (26). These results inferred that miR-218 targeted BMI-1 as a potential tumor suppressor in CRC.

We testified the putative tumor suppressor function of miR-218 in human CRC by both *in vitro* and *in vivo* assays. Overexpression of miR-218 in two colon cancer cell lines (HCT116 and HT29) showed significant growth-suppressing effect by inhibition of cell colony formation. In nude mice, colon cancer cells overexpressing miR-218 displayed a significantly lower growth rate than the control cancer cells. In addition, FACS analysis revealed a significant increase of cell apoptosis in miR-218-reexpressed cells compared with the control cells. Moreover, overexpression of miR-218 can arrest the cell cycle in the G2 phase. Collectively, we indicate for the first time that miR-218 functions as a tumor suppressor in colon cancer. Other reports have shown that miR-218 acts as tumor-suppressive miRNA in several kinds of tumors, including gastric cancer (12), lung cancer (27), cervical cancer (28), head and neck cancer (29) and bladder cancer (16). Our findings have highlighted the importance of miRNA as a potential tumor suppressor in a solid cancer.

The growth-inhibitory activity was associated with rescuing BMI-1-mediated p53 suppression. The p53 protein was upregulated by overexpression of miR-

218 in HCT116 (p53 wild-type) but not in HT29 (p53 mutant). This result was confirmed by enhanced p53 promoter luciferase activity in HCT116 cells after transfection with the miR-218 precursor. p53 is a well-known tumor suppressor gene; inactivation of p53 pathways is a common feature of many cancers (30,31). Other reports have found that BMI-1 inhibits c-Myc-induced apoptosis and c-Myc activates p53-dependent and -independent apoptosis pathways, depending on cancer cell type (32,33). In our investigation, miR-218 mediated BMI-1 downregulation, which is accompanied by an induction of cell apoptosis. These results suggested that miR-218 suppresses BMI-1, which in turn induces apoptosis via a p53-dependent manner.

CDK4 belongs to the Ser/Thr protein kinase superfamily. The complexes formed by CDK4 and the D-type cyclins are involved in the control of cell proliferation and are required for G2 phase cell cycle progression, and the cyclin D3-CDK4 activity is necessary for cell cycle progression through the G2 phase into mitosis (34,35). We demonstrated that miR-218 inhibited BMI-1 downstream target CDK4 protein expression in colon cancer cell lines; thus, miR-218 mediated cell cycle arrest at the G2 checkpoint dependent, in part, on CDK4 inhibition. It was reported that BMI-1 deletion downregulates CDK4 expression (10,36). In this regard, the suppression of BMI-1 may also contribute to the cell cycle arrest at the G2 checkpoint by miR-218.

Recently, studies also demonstrated the tumor-suppressive role of miR-218 in other human cancers. miR-218 was reported to induce caspase-mediated apoptosis through interaction with the mechanistic target of rapamycin (serine/threonine kinase) (mTOR) component Rictor in oral cancer (20) and to activate tumor-suppressive genes Ras association (RalGDS/AF-6) domain family member 1 (RASSF1) and claudin 6 (CLDN6) through directly inhibiting HoxB3 expression in breast cancer (37).

The potential clinical application of miR-218 in human cancer has been reported. Plasma miR-218 was significantly reduced in gastric cancer patients and might potentially serve as a noninvasive biomarker for gastric cancer detection (37). Patients with low miR-218 expression had shorter overall survival than patients with high miR-218 expression in lung cancer (38). However, there is no correlation between miR-218 expression and clinicopathological features in this study. The clinical implication of miR-218 in CRC remains to be investigated in the future with a large-scale sample size.

CONCLUSION

We have identified that miR-218 is downregulated in CRC. miR-218 suppressed colon cancer cell growth and increased cell cycle arrest and apoptosis, at least in part via inhibition of oncogene BMI-1. miR-218 is a candidate tumor-suppressive miRNA of CRC.

ACKNOWLEDGMENTS

This project was supported by a National High-Tech R&D Program in China (863 Program, 2012AA02A506), National Natural Science Foundation of China (81072034) and an ITF fund in Hong Kong (project code ITS/276/11).

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Parkin DM, Bray F, Ferlay J, Pisani P. (2005) Global cancer statistics, 2002. *CA Cancer J. Clin.* 55:74–108.
- Jemal A, et al. (2011) Global cancer statistics. *CA Cancer J. Clin.* 61:69–90.
- Schetter AJ, Harris CC. (2011) Alterations of microRNAs contribute to colon carcinogenesis. *Semin. Oncol.* 38:734–42.
- Wu WK, et al. (2011) MicroRNA in colorectal cancer: from benchtop to bedside. *Carcinogenesis.* 32:247–53.
- Bhatti I, Lee A, Lund J, Larvin M. (2009) Small RNA: a large contributor to carcinogenesis? *J. Gastrointest. Surg.* 13:1379–88.
- Galasso M, Sandhu SK, Volinia S. (2012) MicroRNA expression signatures in solid malignancies. *Cancer J.* 18:238–43.
- Lu J, et al. (2005) MicroRNA expression profiles classify human cancers. *Nature.* 435:834–8.
- Schetter AJ, Okayama H, Harris CC. (2012) The role of microRNAs in colorectal cancer. *Cancer J.* 18:244–52.
- Siddique HR, Saleem M. (2012) Role of BMI-1, a stem cell factor, in cancer recurrence and chemoresistance: reclinical and clinical evidences. *Stem Cells.* 30:372–8.
- Sorrentino BP. (2004) Clinical strategies for expansion of haematopoietic stem cells. *Nat. Rev. Immunol.* 4:878–88.
- Jacobs JJ, et al. (1999) Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev.* 13:2678–90.
- Gao C, et al. (2010) Reduced microRNA-218 expression is associated with high nuclear factor kappa B activation in gastric cancer. *Cancer.* 116:41–9.
- Song L, et al. (2010) miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK- β . *Biochem. Biophys. Res. Commun.* 402:135–40.
- Alajez NM, et al. (2011) MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res.* 71:2381–91.
- Wu DW, Cheng YW, Wang J, Chen CY, Lee H. (2010) Paxillin predicts survival and relapse in non-small cell lung cancer by microRNA-218 targeting. *Cancer Res.* 70:10392–401.
- Tatarano S, et al. (2011) miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. *Int. J. Oncol.* 39:13–21.
- Shivapurkar N, Maitra A, Milchgrub S, Gazdar AF. (2001) Deletions of chromosome 4 occur early during the pathogenesis of colorectal carcinoma. *Hum. Pathol.* 32:169–77.
- Dalloy A, Morton D, Maher ER, Latif F. (2003) SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells. *Cancer Res.* 63:1054–8.
- Alajez NM, et al. (2011) MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res.* 71:2381–91.
- Uesugi A, et al. (2011) The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. *Cancer Res.* 71:5765–78.
- Kang MK, et al. (2007) Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival. *Br. J. Cancer.* 96:126–33.
- Schuringa JJ, Vellenga E. (2010) Role of the polycomb group gene BMI-1 in normal and leukemic hematopoietic stem and progenitor cells. *Curr. Opin. Hematol.* 17:294–9.
- Douglas D, et al. (2008) BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression. *Cancer Res.* 68:6507–15.
- Kim J, Yoon S, Kim C. (2004) The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. *Cancer Lett.* 203:217–24.
- Tateishi K, et al. (2006) Dysregulated expression of stem cell factor BMI-1 in precancerous lesions of the gastrointestinal tract. *Clin. Cancer Res.* 12:6960–6.
- Li DW, et al. (2010) Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer. *J. Cancer Res. Clin. Oncol.* 136:997–1006.
- Davidson MR, et al. (2010) MicroRNA-218 is deleted and downregulated in lung squamous cell carcinoma. *PLoS One.* 5:e12560.
- Martinez I, et al. (2008) Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. *Oncogene.* 27:2575–82.
- Wald AJ, Hoskins EE, Wells SI, Ferris RL, Khan SA. (2011) Alteration of microRNA profiles in squamous cell carcinoma of the head and neck cell lines by human papillomavirus. *Head Neck.* 33:504–12.
- Vogelstein B, et al. (2004) Cancer genes and the pathways they control. *Nat. Med.* 10:789–99.
- Vogelstein B, et al. (2000) Surfing the p53 network. *Nature.* 408:307–10.
- Jacobs JJ, et al. (1999) The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature.* 397:164–8.
- Sakamuro D, et al. (1995) c-Myc induces apoptosis in epithelial cells by both p53-dependent and p53-independent mechanisms. *Oncogene.* 11:2411–8.
- Gabrielli BG, et al. (1999) A cyclin D-Cdk4 activity required for G2 phase cell cycle progression is inhibited in ultraviolet radiation-induced G2 phase delay. *J. Biol. Chem.* 274:13961–9.
- Burgess A, et al. (2006) Inhibition of S/G2 phase CDK4 reduces mitotic fidelity. *J. Biol. Chem.* 281:9987–95.
- Wu J, et al. (2011) Down-regulation of BMI-1 cooperates with artemisinin on growth inhibition of nasopharyngeal carcinoma cells. *J. Cell. Biochem.* 112:1938–48.
- Li Q, Zhu F, Chen P. (2012) miR-7 and miR-218 epigenetically control tumor suppressor genes RASSF1A and Claudin-6 by targeting HoxB3 in breast cancer. *Biochem. Biophys. Res. Commun.* 424:28–33.
- Wu DW, Cheng YW, Wang J, Chen CY, Lee H. (2010) Paxillin predicts survival and relapse in non-small cell lung cancer by microRNA-218 targeting. *Cancer Res.* 70:10392–401.