

Epigenetically silenced microRNAs in gastric cancer: Functional analysis and identification of their target genes

JIN-HAN BAE^{1*}, MYOUNG JOO KANG^{2*}, KWANG-MO YANG¹, TAE-OH KIM² and JOO MI YI¹

¹Research Center, Dongnam Institute of Radiological and Medical Sciences (DIRAMS), Busan 619-953; ²Department of Internal Medicine, Inje University Haeundae Paik Hospital, Busan 612-896, Republic of Korea

Received April 2, 2015; Accepted May 11, 2015

DOI: 10.3892/or.2015.4036

Abstract. microRNAs (miRNAs), which are small non-coding RNA molecules, can participate in diverse biological functions and act as oncogenes or tumor suppressors by inhibiting target gene expression. The alteration of miRNA expression is observed in many types of human cancers and has been implicated in carcinogenesis. Since miRNAs have been known to be downregulated in most cancer types, there is growing evidence that several miRNAs are downregulated by DNA hypermethylation. Here, we determined that *MIR219.2*, *MIR663B* and *MIR1237* were transcriptionally silenced by DNA hypermethylation in human gastric cancer cell lines. Moreover, we demonstrated the functional roles of these epigenetically silenced miRNAs by ectopically expressing them in gastric cancer cells, which caused the suppression of growth and proliferation. In addition, wound closure, cell migration, and invasion were significantly reduced in AGS cells following transfection with *MIR219.2*, *MIR663B* or *MIR1237* mimics. Notably, epithelial-to-mesenchymal transition (EMT)-associated proteins were decreased in response to ectopic expression of these miRNAs, supporting the notion that these miRNAs have a tumor-suppressive effect in gastric cancer. We finally predicted the targets of these miRNAs and identified several candidate genes, the expression levels of which were significantly downregulated by ectopic expression of *MIR219.2*, *MIR663B* or *MIR1237* mimics in the

gastric cancer cell lines. Our study provides strong evidence that these miRNAs are transcriptionally regulated by DNA methylation in gastric cancer and have tumor-suppressive roles by decreasing the mesenchymal traits in cancer as well as by targeting cancer-associated genes.

Introduction

microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally by binding to complementary sequences in the 3'-untranslated region of messenger RNAs (mRNAs) (1). Approximately 30% of human genes are under miRNA regulation. Not only does each miRNA regulate many genes, but also each gene is regulated by many miRNAs (2,3). Many miRNAs are highly conserved among species and play critical roles in a variety of biological processes, including development, differentiation, proliferation, apoptosis and stem cell maintenance (1,4,5). Consistent with their roles in these processes, many studies have shown widespread alteration of miRNA expression patterns in cancer (6,7). Indeed, downregulation of a subset of miRNAs is a commonly observed feature noted in cancer, suggesting that these molecules may act as tumor suppressors (8). Many other recent studies show that dysfunction of miRNAs is related to chronic lymphocytic leukemia and several solid tumors, including colon, lung, breast, gastric and liver cancers (1,9-12). Furthermore, disruption of miRNA biogenesis in various types of cancer has been implicated in a variety of mechanisms, such as genomic deletion, mutation, epigenetic silencing and miRNA processing defects (13-16).

Gastric cancer is one of the most common causes of death from cancer among men and women worldwide (17). To date, we and many other researchers have identified a wide variety of tumor-suppressor genes and other tumor-related genes that are silenced by abnormal DNA methylation in gastric cancer (18,19). In addition, it was recently shown that epigenetic mechanisms are involved in the alteration of several miRNA genes in gastric cancer (18,20), although little is known concerning the dysregulation of miRNAs in this disease. Previously, several novel DNA methylation-regulated miRNAs were classified in colon cancer cells by combining genome-wide DNA methylation and miRNA expression analyses (21). In the present study, we aimed to test the

Correspondence to: Dr Joo Mi Yi, Research Center, Dongnam Institute of Radiological and Medical Sciences (DIRAMS), Busan 619-953, Republic of Korea
E-mail: jmyi@dirams.re.kr

Dr Tae-Oh Kim, Department of Internal Medicine, Inje University Haeundae Paik Hospital, Busan 612-896, Republic of Korea
E-mail: kto0440@paik.ac.kr

*Contributed equally

Key words: DNA methylation, 5-aza-2'-deoxycytidine, microRNA, gastric cancer, tumor-suppressive effect

epigenetic silencing of three miRNAs (*MIR219.2*, *MIR663B* and *MIR1237*) in gastric cancer and evaluate the phenotypic impact of such epigenetic silencing on gastric cancer.

In short, we found *MIR219.2*, *MIR663B* and *MIR1237* to harbor dense DNA methylation in gastric cancer cell lines and that such aberrant DNA hypermethylation correlated with their transcriptional silencing as well as re-expression after 5-aza-2'-deoxycytidine (5-aza-dC) treatment. We also determined that ectopic expression of *MIR219.2*, *MIR663B* and *MIR1237* resulted in important biological roles, such as a decrease in cell proliferation, migration and invasion, suggesting that these miRNAs have a tumor-suppressive role in gastric cancer by decreasing epithelial-to-mesenchymal transition (EMT)-associated factors. In addition, we retrieved several target genes regulated by *MIR219.2*, *MIR663B* and *MIR1237* using TargetScan and miRBase databases. Finally, we identified the candidate target genes regulated by *MIR219.2*, *MIR663B* and *MIR1237*, suggesting that epigenetic silencing of these miRNAs may be responsible for some important tumor-suppressive characteristics of gastric cancer cells.

Materials and methods

Cell culture and 5-aza-dC treatments. Five human gastric cancer cell lines (AGS, AZ521, KATO III, NCI-N87 and SNU-1) were used in this study and were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Korea Research Institute of Bioscience and Biotechnology BioResource Center in Korea. The AGS cell line was cultured using Ham's F-12, AZ521 was maintained in MEM medium, KATO III was grown in Iscove's modified Dulbecco's medium (IMDM), and NCI-N87 and SNU-1 were propagated in RPMI-1640 medium (all from WelGene, Daegu, Korea). All cell culture media were supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% antibiotic-antimycotic (Gibco, Grand Island, NY, USA). All cell lines were incubated at 37°C with 20% O₂ and 5% CO₂. To investigate the effect of 5-aza-dC treatments, cells were treated with 5 μM 5-aza-dC (Sigma, St. Louis, MO, USA) for 72 h.

Tissue samples. The biospecimens and data used in this study were provided by the Inje Biobank of Inje University Busan Paik Hospital, a member of the Korea Biobank Network.

DNA methylation analyses. Primer pairs for methylation analysis were preferentially designed near the putative transcription start site (TSS) in the 5' CpG islands of the genes. All methylation-specific primers were designed by MethPrimer (<http://www.urogene.org/methprimer>). Methylation analyses, including methylation-specific PCR (MSP) and bisulfite sequencing analysis, were performed as previously described (18).

Quantitative real-time RT-PCR (qRT-PCR). Total RNA was isolated from human gastric cancer cell lines using TRI-Solution (Bioscience Technology, Kyungsan, Korea) following the manufacturer's instructions. RNA quantity was measured using a NanoDrop 2000/2000c instrument (Thermo Scientific, Rockford, IL, USA), and 1 μg of RNA

was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). For expression studies using RT-PCR, primers were designed using the Open Access Primer3 program (<http://frodo.wi.mit.edu/primer3>). Quantitative RT-PCR was performed on a C1000 Thermal Cycler (BioRad) using the PCR primers we specifically designed. The expression levels of pri-miRNAs and target genes were normalized to β-actin levels. ΔΔCt method was used for the relative quantification of expression.

miRNA transfection. To ectopically express *MIR219.2*, *MIR663B* and *MIR1237* in the AGS gastric cancer cell line, cells were transfected with 20 nM hsa-miR-219a-2-3p (MSY0004675), hsa-miR-663b (MSY0005867) and hsa-miR-1237 (MSY0005592) miScript mimics, or AllStars Negative Control siRNA (1027281) (all from Qiagen, GmbH, Hilden, Germany) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell proliferation and viability assays. Cell proliferation was analyzed using the MTT assay. At 24 h after transfection of miRNA mimics or the negative control, AGS cells (2x10⁵ cells/well) were re-plated in 6-well plates and incubated at 37°C. After 72 h, the cells were washed twice with phosphate-buffered saline (PBS) and 5 mg/ml MTT in PBS was added to each well for 4 h. After removing the MTT solution, a solubilization solution (DMSO/EtOH, 1:1 ratio) was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was measured using a Paradigm microplate reader (Beckman Coulter, Fullerton, CA, USA). After re-plating (5x10³ cells/well) in 6-well plates, cell numbers were counted at 24, 48, 72 and 96 h.

Wound-healing assay. Mimic or control siRNA-transfected AGS cells were plated overnight to achieve a subconfluent cell monolayer in 6-well plates. Then, a scratch was made in the cell layer with a sterile 200-μl pipette tip, and cultures were washed twice with serum-free medium to remove floating cells. Cells were incubated in culture medium. After 16 h, wound healing was visualized by comparing images using a QImaging QIClick camera system mounted on a phase-contrast Nikon TS100 microscope (Nikon, Melville, NY, USA). The distance traveled by the cells was determined by measuring the wound width at 16 h and subtracting it from the wound width at 0 h.

Migration and invasion assays. Cell migration was determined using Transwell plates (24-well, 8-μm pore size; Corning Costar, Rochester, NY, USA) and the invasion assay was carried out using a Matrigel-coated invasion chamber (24-well, 8-μm pore size; Corning Costar). The upper chamber contained cells in specific medium with 1% FBS, and the lower chamber contained medium with 10% FBS. Cells were incubated for 16 h at 37°C with 20% O₂ and 5% CO₂. Non-migratory or non-invasive cells were scraped off the upper membrane with a cotton swab. Migratory or invasive cells remaining on the bottom membrane were counted after staining with Giemsa (Sigma). Images were captured using a QIcam image camera system mounted on a Nikon Eclipse 80i microscope (Nikon).

Western blot analysis. Total cell lysates (20 μ g) were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were blocked with 5% milk dissolved in Tris-buffered saline containing 0.02% Tween-20 and incubated overnight at 4°C with specific primary antibodies. The membranes were subsequently incubated with specific horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using a Fusion FX5 system (Vilber Lourmat, Eberhardzell, Germany). The following primary antibodies were used: anti-E-cadherin, anti-N-cadherin, anti-Slug and anti-vimentin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti- β -actin (Sigma) antibodies.

Bioinformatic analysis of miRNA target genes. The miRNA target genes were collected from miRBase (<http://www.miRbase.org>) and TargetScan Human Release 6.2 (<http://www.targetscan.org>). Sequences of miRNAs were aligned using the BioEdit program (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>), and, using the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>) and BLAT search, genome locations and matches with CpG islands were analyzed. To identify the functions and signaling pathways of the target genes, the super-pathway categories within the GeneCards database (<http://www.genecards.org/cgi-bin>), which included Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.kegg.jp/kegg/pathway.html>), were used. Gene interaction analyses of miRNA target genes were performed using the GeneMANIA web tool (<http://www.genemania.org>) and visualized by Cytoscape ver. 3.0.2. Output of the GeneMANIA search followed query-dependent weighting options.

Statistical analyses. Quantified data are expressed as the mean \pm standard deviation (SD) values. Significance testing was conducted via Student's t-test.

Results

Expression of *MIR219.2*, *MIR663B* and *MIR1237* are regulated by DNA hypermethylation in gastric cancer cell lines. To investigate whether *pri-MIR219.2*, *pri-MIR663B* and *pri-MIR1237* were regulated by DNA methylation in gastric cancer, we treated AGS, AZ521, N87, KATO III and SNU-1 gastric cancer cells with the demethylating agent 5-aza-dC to determine whether these miRNAs are re-expressed after 5-aza-dC treatment by qRT-PCR.

Notably, we observed the re-expression of three miRNAs after 5-aza-dC treatment in only AGS cells; we did not observe any significant re-expression of these miRNAs after 5-aza-dC treatment in the other cell lines that were tested (Fig. 1). To verify whether these miRNAs are upregulated by DNA methylation changes, we assessed the level of DNA methylation in the proximal region of these mRNAs by MSP in gastric cancer cell lines and bisulfite genomic sequencing in AGS cells.

Using specific primers in their CpG island region for methylation analyses (Fig. 2A), we evaluated the methylation patterns in five different gastric cancer cell lines. *MIR219.2* and *MIR663B* were completely methylated in most of the

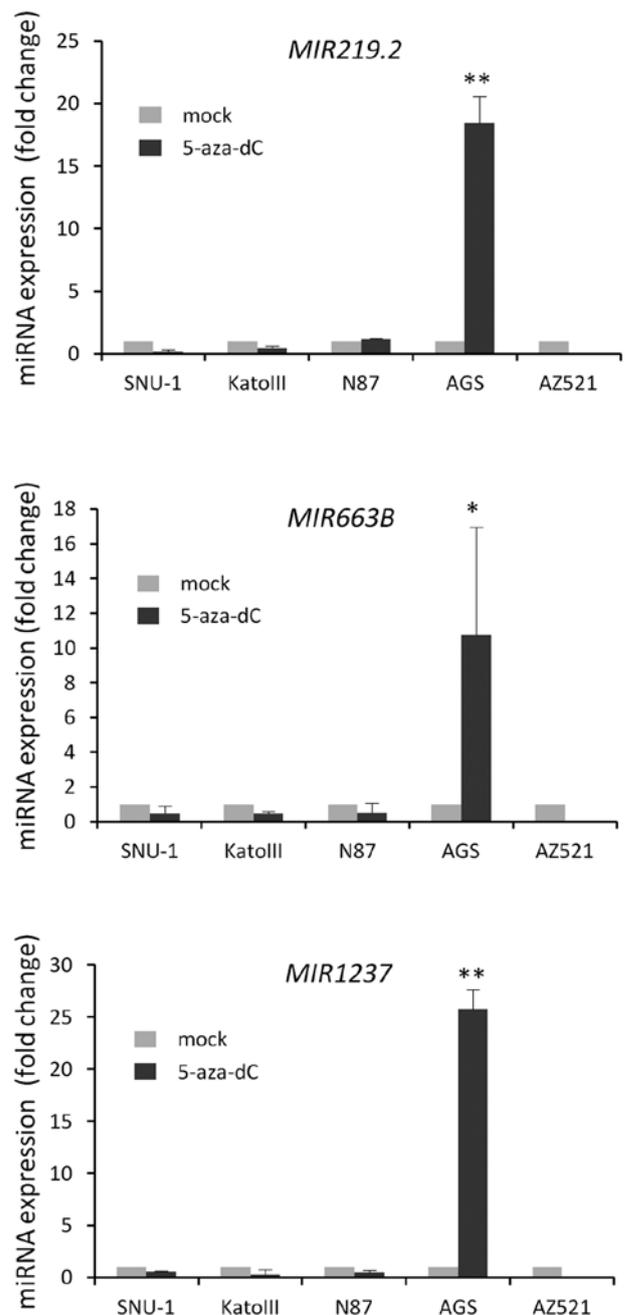


Figure 1. Analysis of *MIR219.2*, *MIR663B* and *MIR1237* expression in gastric cancer cell lines. Quantitative RT-PCR was performed to assess *pri-miRNAs* levels in gastric cancer cell lines (AGS, AZ521, KATO III, NCI-N87 and SNU-1) before and after 5 μ M 5-aza-dC treatment for 72 h. Dark gray bars indicate 5-aza-dC treatment. Relative *pri-miRNA* levels are shown as mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$, indicate a significant increase in *pri-miRNA* expression after 5-aza-dC treatment.

gastric cancer cell lines that were tested. *MIR1237* was mostly methylated with a partial methylation pattern compared with the other miRNAs (Fig. 2B). However, we confirmed that these miRNAs are mostly methylated in gastric cancer cell lines, suggesting that these data correlate with the re-expression of miRNAs by 5-aza-dC treatment. We also confirmed the methylation status in the AGS cell line by bisulfite sequencing analysis, showing that most CpG sites in these miRNAs were methylated (Fig. 2C). To further detect whether *MIR219.2*,

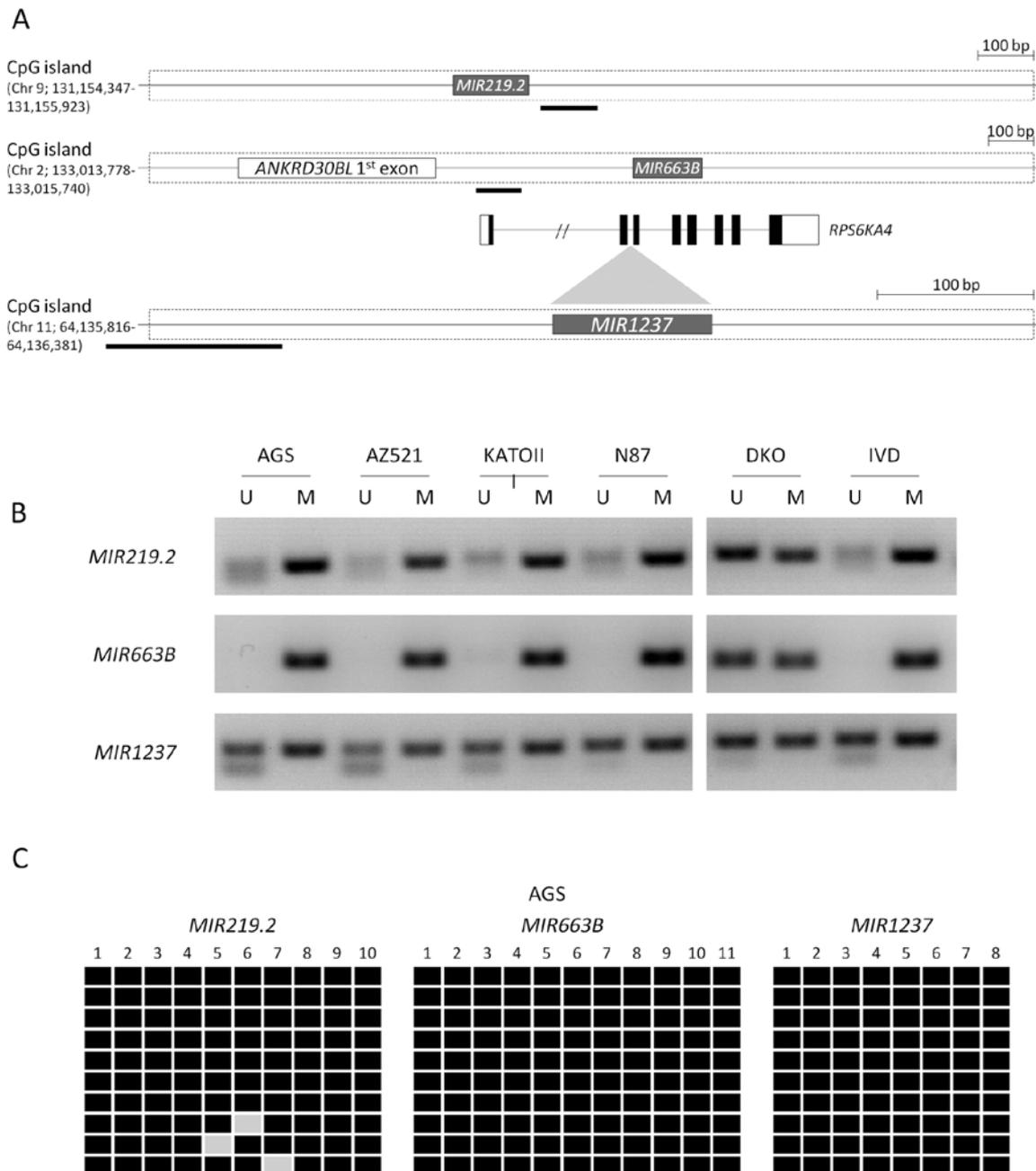


Figure 2. Methylation analyses of *MIR219.2*, *MIR663B* and *MIR1237* in gastric cancer cell lines. (A) Schematic representation of *MIR941* and *MIR1247* CpG islands (dotted boxes). Three miRNAs are embedded in the CpG islands (gray boxes). The regions for MSP and bisulfite sequencing analyses are indicated by black bars below the CpG islands. (B) MSP analysis of *MIR219.2*, *MIR663B* and *MIR1237* in four gastric cancer cell lines. *In vitro* methylated DNA (IVD), and both DNMT1 and DNMT3B double knockout HCT116 (DKO) cells were used as positive and negative controls, respectively. U and M indicate unmethylated and methylated signals, respectively. (C) Representative bisulfite sequencing analysis was carried out for *MIR219.2*, *MIR663B* and *MIR1237* in the AGS gastric cancer cell line. Each box represents a CpG dinucleotide. Black boxes represent methylated cytosines, while gray boxes represent unmethylated cytosines.

MIR663B and *MIR1237* are methylated in gastric cancer patient tissues, we examined the methylation status of these miRNAs in five gastric cancer primary tissues using bisulfite sequencing analysis. We observed that *MIR219.2*, *MIR663B* and *MIR1237* were highly methylated in the gastric cancer primary tissues with 95% (*MIR219.2* and *MIR663B*) and 83% (*MIR1237*) of the analyzed CpG sites methylated (Fig. 3). These results suggest that *MIR219.2*, *MIR663B* and *MIR1237* are densely methylated in gastric cancer cells correlated with transcriptional silencing as well as they are also highly methylated in cancer specimens.

Functional analysis of MIR219.2, MIR663B and MIR1237 in gastric cancer cells. We next tested the biological functions of these epigenetically regulated miRNAs to understand the relevance of their DNA methylation in gastric cancer cells. To determine whether expression of these epigenetically silenced miRNAs would affect cancer cell growth and migration, we transfected AGS cells with miRNA mimics of *MIR219.2*, *MIR663B*, *MIR1237* and a non-targeting negative control. Growth curve analysis showed that AGS cell growth was inhibited by ~63% (*MIR219.2*), 68% (*MIR663B*) and 69% (*MIR1237*) compared to the control (Fig. 4A). Along with growth data,

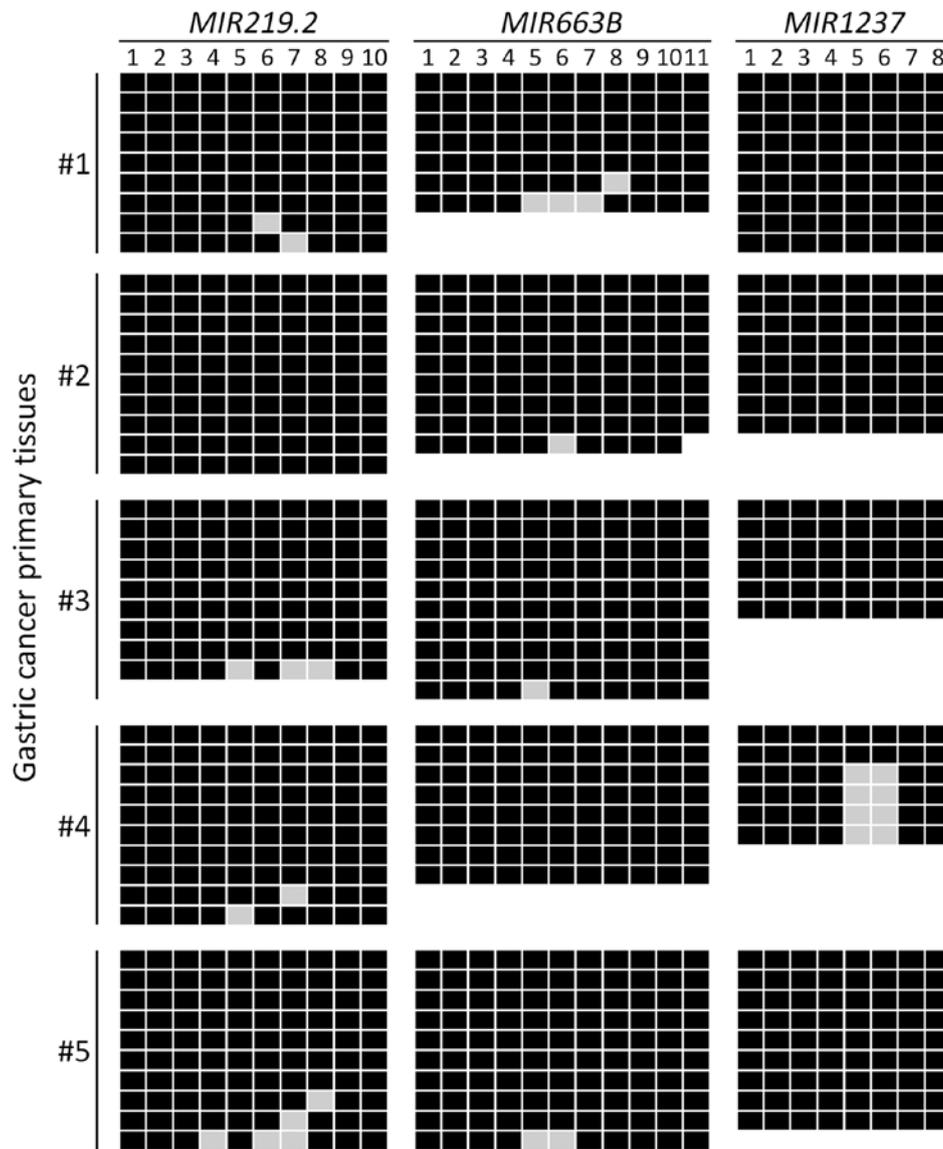


Figure 3. Methylation levels of *MIR219.2*, *MIR663B* and *MIR1237* in gastric cancer patient primary tissues. Representative bisulfite sequencing analysis was carried out for *MIR219.2*, *MIR663B* and *MIR1237* in five gastric adenocarcinomas. Each box represents a CpG dinucleotide. Black boxes represent methylated cytosines, while gray boxes represent unmethylated cytosines.

MTT assay demonstrated a 13, 11 and 10% decrease in proliferation following *MIR219.2*, *MIR663B* and *MIR1237* mimic transfection compare to the control, respectively (Fig. 4B).

Based on the growth curve, we postulated that ectopic expression of *MIR219.2*, *MIR663B* and *MIR1237* would impact cell migration or invasion. In the wound-healing assays, AGS cells transfected with each miRNA mimic showed less wound closure compared with the mock-treated or control cells (Fig. 4C). These results were further confirmed using Transwell migration and invasion assays (Fig. 4D and E). These data indicate that ectopic expression of these miRNAs could affect actual migration- or invasion-associated molecules in cancer. Tumor cell invasion is involved with the loss of cell-cell interaction together with the acquisition of migratory properties and is often associated with EMT (22).

Therefore, we next examined whether ectopic expression of these miRNAs suppresses the migratory and invasive properties of gastric cancer cells through disturbing EMT.

Ectopic expression of miRNAs in the AGS cells decreased mesenchymal cell markers, such as N-cadherin and vimentin, while increasing epithelial cell marker E-cadherin in the AGS cells. These EMT markers are directly regulated by EMT transcription factors, such as Slug. Notably, Slug expression was markedly decreased by ectopic expression of *MIR219.2*, *MIR663B* and *MIR1237* (Fig. 4F). These data are consistent with the migration and invasion data and strongly support the hypothesis that miRNAs could have a tumor-suppressive effect in gastric cancer by suppressing mesenchymal traits of cancer.

Identification of target genes regulated by MIR219.2, MIR663B and MIR1237. miRNAs modulate gene expression by interacting with their target mRNAs, resulting in mRNA degradation or translational repression. To help us identify potential target genes of *MIR219.2*, *MIR663B* and *MIR1237* in gastric cancer, target genes were predicted using TargetScan Human 6.2 and miRBase. For *MIR219.2*, *MIR663B* and

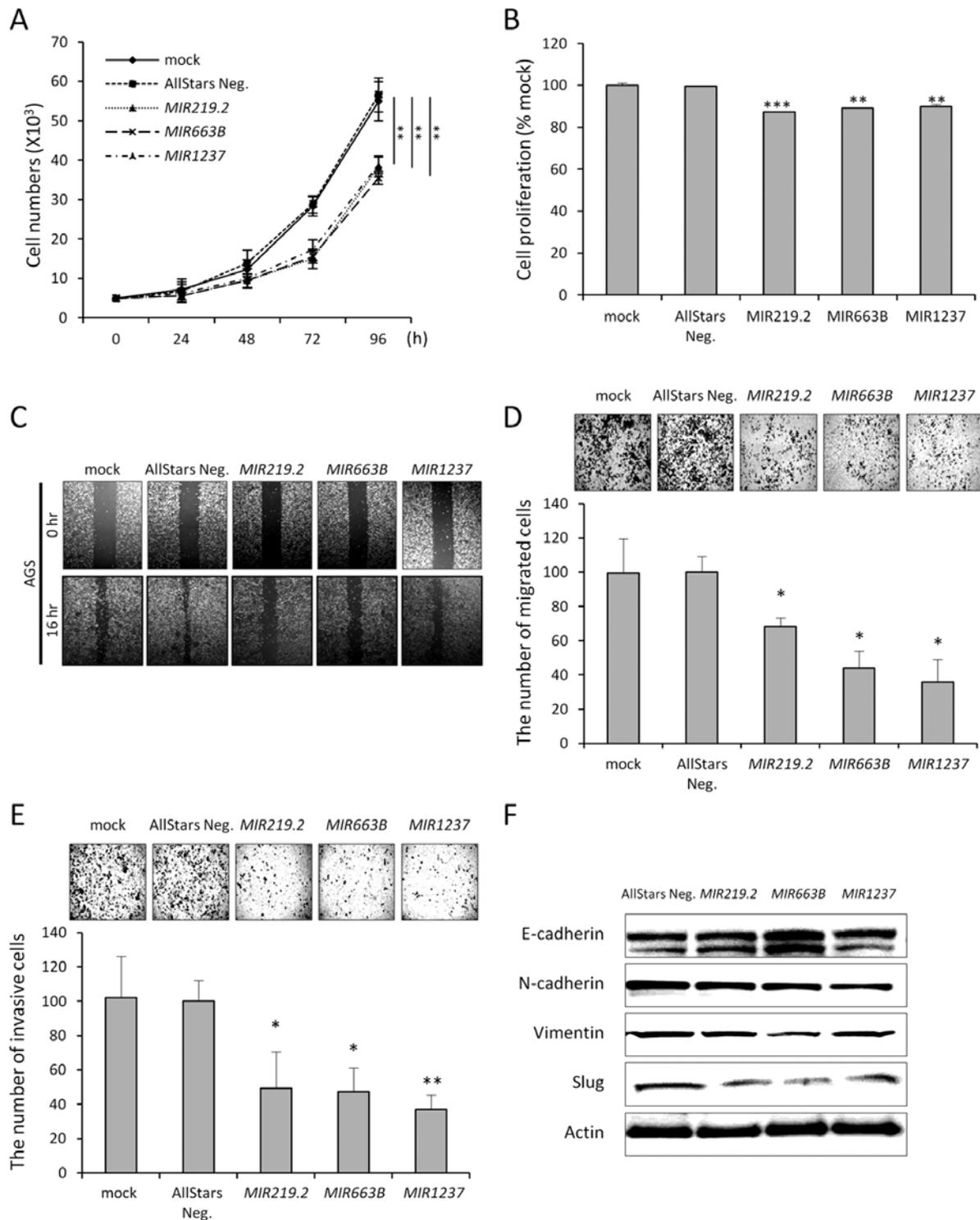


Figure 4. Functional analyses of *MIR219.2*, *MIR663B* and *MIR1237* in AGS cells. (A) Growth curves and (B) MTT assays of AGS mock-treated cells and AGS cells transfected with non-targeting negative control miRNA (AllStars neg.) or *MIR219.2*, *MIR663B* or *MIR1237* mimics. (C) Wound-healing assay for AGS cells transfected with mock, non-targeting negative controls miRNA (AllStars neg.) or *MIR219.2*, *MIR663B* or *MIR1237* mimics. Images were captured at 16 h after wounding. (D) Transwell migration and (E) invasion assays using AGS mock-treated cells and AGS cells transfected with non-coding negative control miRNA or *MIR219.2*, *MIR663B* or *MIR1237* mimics. Representative fields of migratory and invasive cells on the membrane are shown. The bar graph represents the mean of three random microscopic fields per membrane, and the error bars represent the standard deviations (SDs). (F) Western blot analysis for the expression of EMT-associated proteins (E-cadherin, N-cadherin, vimentin and Slug) in *MIR219.2*, *MIR663B* or *MIR1237* mimic-transfected AGS cells compared with non-targeting negative control miRNA transfection.

MIR1237, 156, 136 and 299 target genes were predicted, respectively. Since many target genes were identified, we selected several common genes for the three miRNAs (Table I). Therefore, to validate whether these candidate target genes are

regulated by miRNAs, we analyzed the mRNA levels of these target genes using qRT-PCR in the AGS cells upon transfection of the miRNA mimics. We found that *MIR219.2*, *MIR663B* and *MIR1237* mimics consistently depleted mRNA levels

Table I. Target gene list for *MIR219-2*, *MIR663B* and *MIR1237*.

Gene	miRNA			Accession no.	Function
	219.2	663B	1237		
<i>ANKRD13C</i>	V		V	NM_030816.4	Ankyrin repeat domain 13C
<i>C20orf112</i>		V	V	NM_001256798.1; NM_080616.4	Chromosome 20 open reading frame 112
<i>CBX5</i>		V	V	NM_001127321.1; NM_001127322.1; NM_012117.2	Chromobox homolog 5
<i>CCDC113</i>		V	V	NM_001142302.1; NM_014157.3	Coiled-coil domain containing 113
<i>CCND2</i>		V	V	NM_001759.3	Cyclin D2
<i>CLCN5</i>		V	V	NM_000084.4; NM_001127898.3; NM_001127899.3; NM_001272102.1; NM_001282163.1	Chloride channel, voltage-sensitive 5
<i>CNNM1</i>		V	V	NM_020348.2	Cyclin M1
<i>CT62</i>	V		V	NM_001102658.1	Cancer/Testis antigen 62
<i>CUX1</i>		V	V	NM_001202543.1; NM_001202544.1; NM_001202545.1; NM_001202546.1; NM_001913.3; NM_181500.2; NM_181552.3	Cut-like homeobox 1
<i>DCC</i>	V		V	NM_005215.3	Deleted in colorectal carcinoma
<i>DCX</i>	V	V		NM_000555.3; NM_001195553.1; NM_178151.2; NM_178152.2; NM_178153.2	Doublecortin
<i>ERBB3</i>	V		V	NM_001005915.1; NM_001982.3	V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 3
<i>IPO9</i>	V		V	NM_018085.4	Importin 9
<i>NFIA</i>	V		V	NM_001134673.3; NM_001145511.1; NM_001145512.1; NM_005595.4	Nuclear factor I/A
<i>NFIB</i>	V		V	NM_001190737.1; NM_001190738.1; NM_001282787.1; NM_005596.3	Nuclear factor I/B
<i>NPEPPS</i>	V		V	NM_006310.3	Aminopeptidase puromycin sensitive
<i>PMEPA1</i>	V		V	NM_001255976.1; NM_020182.4; NM_199169.2; NM_199170.2; NM_199171.2	Prostate transmembrane protein, androgen induced 1
<i>PTPRT</i>	V		V	NM_007050.5; NM_133170.3	Protein tyrosine phosphatase, receptor type, T
<i>PURB</i>	V		V	NM_033224.4	Purine-rich element binding protein B
<i>RC3H1</i>	V		V	NM_172071.2	Ring finger and CCCH-type domains 1
<i>SERP1</i>	V		V	NM_014445.3	Stress-associated endoplasmic reticulum protein 1
<i>SGIP1</i>	V		V	NM_032291.2	SH3-domain GRB2-like (endophilin) interacting protein 1
<i>SMC1A</i>		V	V	NM_001281463.1; NM_006306.3	Structural maintenance of chromosomes 1A
<i>STAT5B</i>		V	V	NM_012448.3	Signal transducer and activator of transcription 5B
<i>USP47</i>	V	V		NM_001282659.1; NM_017944.3	Ubiquitin-specific peptidase 47
<i>WASL</i>	V		V	NM_003941.3	Wiskott-Aldrich syndrome-like
<i>YOD1</i>	V		V	NM_001276320.1; NM_018566.3	YOD1 OTU deubiquinating enzyme 1 homolog

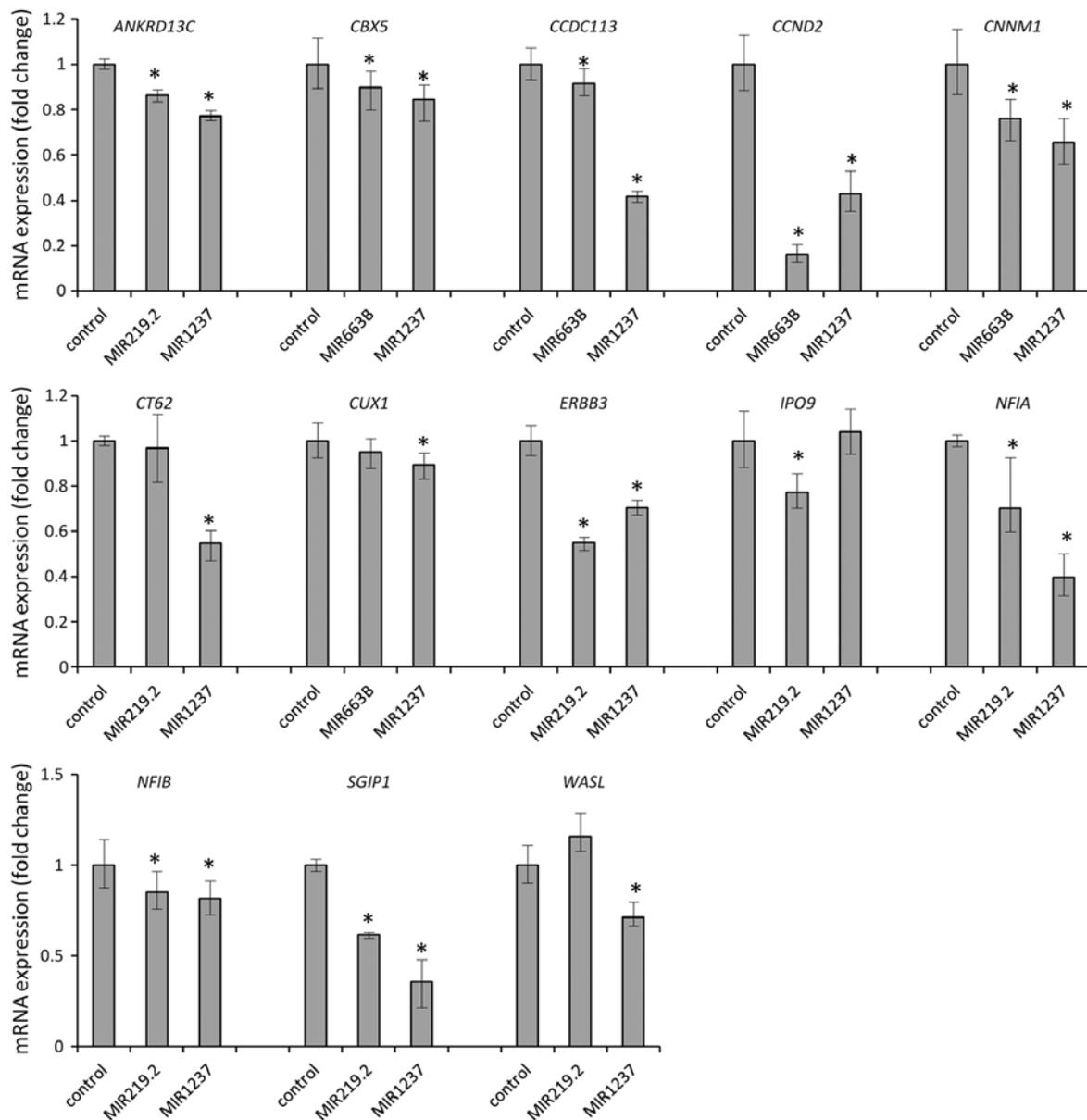


Figure 5. Candidate target genes are downregulated by *MIR219.2*, *MIR663B* or *MIR1237* mimics. Quantitative RT-PCR was carried out in the AGS cell line transfected with non-targeting negative control miRNA (AllStars neg.) or *MIR219.2*, *MIR663B* or *MIR1237* mimics. * $P < 0.05$, indicates statistically significant decrease of gene expression levels compared to control.

of *ANKRD13C*, *CBX5*, *CCDC113*, *CCND2*, *CNNM1*, *CT62*, *CUX1*, *ERBB3*, *IPO9*, *NFIA*, *NFIB*, *SGIP1* and *WASL* (Fig. 5). After we validate this, we identified 13 candidates as bona fide targets of *MIR219.2*, *MIR663B* and *MIR1247* in gastric cancer. All 13 candidate genes were downregulated by ectopic expression of *MIR219.2*, *MIR663B* and *MIR1247* in the AGS cells which suggest that expression of the target genes we identified is regulated by these miRNAs.

Discussion

miRNAs are important regulatory molecules that modulate gene expression in both developmental and disease processes. Their regulation is, therefore, a critical component in understanding each biological context (23). Dysregulation of miRNA expression is commonly observed in a wide

variety of cancers, and epigenetic mechanisms have been shown to be key mediators underlying the downregulation of miRNA expression. To screen for epigenetically silenced miRNA genes in gastric cancer, we first performed a microarray analysis to identify miRNAs upregulated by demethylation and histone deacetylase inhibition. There are several miRNAs that have been found to be regulated by DNA hypermethylation and could have tumor-suppressive roles in various cancers.

In recent years, accumulated evidence that the transcription of a handful of miRNAs in cancer is regulated by DNA methylation has been reported. For example, *MIR127* has been found to be activated by epigenetic drug treatment in cancer cells (15). In addition, methylation of *MIR9* and *MIR148a* has been observed in human metastatic cancer cell lines (24), and *MIR203* is epigenetically silenced in hematopoietic

malignancies, which leads to enhanced expression of ABL1 and BCR-ABL1 (25). Recently, *MIR34a* reportedly acts as a tumor suppressor in colon cancer and neuroblastoma (25,26). All three *MIR34* family members are directly regulated by p53, and ectopic expression of *MIR34s* induces cell cycle arrest and/or apoptosis in human cancer cells (5,27,28). Conversely, expression of *MIR34s* is frequently down-regulated in human malignancies, including lung, colon and ovarian cancers (26-29). Very recently we also first reported that transcription of *MIR941*, *MIR1247* and their target genes are regulated by DNA methylation in gastric cancers (18). In addition, Lujambio *et al* (24) identified *MIR34b/c* methylation by screening cell lines derived from metastatic colon cancer, melanoma, and head and neck cancer, and Corney *et al* (28) recently reported downregulation of *MIR34b/c* in metastatic ovarian cancer, which suggests that inactivation of *MIR34b/c* may be associated with cancer metastasis.

In the present study, ectopic expression of *MIR219.2*, *MIR663B* and *MIR1237* reduced cell growth, proliferation, migration and invasion, suggesting that these miRNAs have tumor-suppressive effects in gastric cancer cells. Moreover, we support more strongly a tumor-suppressive role as evidenced by decreased mesenchymal phenotypes using mesenchymal cell markers, such as N-cadherin and vimentin.

We predicted target genes using the TargetScan and miRBase databases and found 13 candidate genes that were validated by qRT-PCR among the more than 100 predicted target genes. These 13 genes could be potential targets of *MIR219.2*, *MIR663B* and *MIR1237*. Although these genes were among the predicted targets, we were interested in how these genes are associated within the cellular pathway network. Networks of collected target genes were analyzed together by GeneMANIA and visualized by Cytoscape version 3.0.2. Twenty-four target genes were found to be related to 20 neighbor genes, and there are 170 total interaction links. In particular, the functions of networks are categorized as follows: cohesin complex, protein-DNA complex, regulation of anion transport, chloride channel activity, chromosome condensation, anion channel activity, chloride transmembrane transporter activity, excretion, chloride transport and nuclear chromosome (data not shown). These results support the hypothesis that selected target genes may play various important roles and are intimately connected with each other. Therefore, to understand the possible roles of selected target genes, signal pathways and the relationship with cancers were predicted using KEGG and GeneCards databases. These data suggest that target genes of *MIR219.2*, *MIR663B* and *MIR1237* are related to each other through critical cancer pathways; therefore, further study is necessary to understand the biological interactions between miRNAs and target genes in cancer.

Among the candidate target genes of *MIR219.2* and *MIR1237*, the receptor tyrosine kinase *ERBB3* (epidermal growth factor receptor family) has been reported to be targeted by several miRNAs (*MIR199a/125b*, *MIR143* and *MIR145*) in various types of cancer (30-32). High levels of *ERBB3* are strongly associated with tumor progression and poor outcome in gastric cancer patients (33,34). *CCND2* (cyclin D2) is known as a proto-oncogene, and overexpression of *CCND2* is closely correlated with progression in gastric cancer (35,36). Similar

to our study, *CCND2* was found to be downregulated by *MIR1*, and then cell proliferation, migration and invasion were reduced in thyroid cancer carcinoma cells (37). In addition, *CCND2* is also targeted by *MIR206* and *MIR29*, which results in the suppression of cell growth in gastric cancer (38,39).

In summary, we demonstrated that *MIR219.2*, *MIR663B* and *MIR1237* are silenced in gastric cancer cell lines by DNA hypermethylation. Our expression and functional studies suggest that ectopic expression of these miRNAs has tumor-suppressive effects in the AGS gastric cancer cell line by decreasing mesenchymal phenotypes. Moreover, their molecular targets are associated with multiple biological pathways according to our bioinformatic analyses. Therefore, our functional data on *MIR219.2*, *MIR663B* and *MIR1237* target genes is a compelling lead, but the detailed relationships between many more target genes and the three miRNAs will require further study. More importantly, we demonstrated that such abnormal epigenetic silencing occurs in gastric cancer and may be useful in developing new potential therapeutic tools for this disease.

Acknowledgements

This study was supported by the National R&D Program (nos. 50591-2015) through the Dongnam Institute of Radiological and Medical Sciences (DIRAMS) funded by the Ministry of Science, ICT and Future Planning (MSIP) of the Korean government.

References

- Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP and Burge CB: Prediction of mammalian microRNA targets. *Cell* 115: 787-798, 2003.
- Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15-20, 2005.
- He L and Hannon GJ: MicroRNAs: Small RNAs with a big role in gene regulation. *Nat Rev Genet* 5: 522-531, 2004.
- He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, *et al*: A microRNA component of the p53 tumour suppressor network. *Nature* 447: 1130-1134, 2007.
- Calin GA and Croce CM: MicroRNA signatures in human cancers. *Nat Rev Cancer* 6: 857-866, 2006.
- Dalmay T and Edwards DR: MicroRNAs and the hallmarks of cancer. *Oncogene* 25: 6170-6175, 2006.
- Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, *et al*: MicroRNA expression profiles classify human cancers. *Nature* 435: 834-838, 2005.
- Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, *et al*: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 64: 3753-3756, 2004.
- Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, *et al*: MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 65: 7065-7070, 2005.
- Yoon SO, Chun SM, Han EH, Choi J, Jang SJ, Koh SA, Hwang S and Yu E: Deregulated expression of microRNA-221 with the potential for prognostic biomarkers in surgically resected hepatocellular carcinoma. *Hum Pathol* 42: 1391-1400, 2011.
- Mraz M and Pospisilova S: MicroRNAs in chronic lymphocytic leukemia: From causality to associations and back. *Expert Rev Hematol* 5: 579-581, 2012.

13. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, *et al*: Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 99: 15524-15529, 2002.
14. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, *et al*: A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* 353: 1793-1801, 2005.
15. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA and Jones PA: Specific activation of microRNA-127 with down-regulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 9: 435-443, 2006.
16. Nakamura T, Canaani E and Croce CM: Oncogenic All1 fusion proteins target Drosha-mediated microRNA processing. *Proc Natl Acad Sci USA* 104: 10980-10985, 2007.
17. Parkin DM: Global cancer statistics in the year 2000. *Lancet Oncol* 2: 533-543, 2001.
18. Kim JG, Kim TO, Bae JH, Shim JW, Kang MJ, Yang K, Ting AH and Yi JM: Epigenetically regulated MIR941 and MIR1247 target gastric cancer cell growth and migration. *Epigenetics* 9: 1018-1030, 2014.
19. Ushijima T, Nakajima T and Maekita T: DNA methylation as a marker for the past and future. *J Gastroenterol* 41: 401-407, 2006.
20. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T and Ushijima T: DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. *Int J Cancer* 124: 2367-2374, 2009.
21. Yan H, Choi AJ, Lee BH and Ting AH: Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. *PLoS One* 6: e20628, 2011.
22. Thiery JP: Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2: 442-454, 2002.
23. Esquela-Kerscher A and Slack FJ: OncomiRs - microRNAs with a role in cancer. *Nat Rev Cancer* 6: 259-269, 2006.
24. Lujambio A, Calin GA, Villanueva A, Ropero S, Sánchez-Céspedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, *et al*: A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci USA* 105: 13556-13561, 2008.
25. Welch C, Chen Y and Stallings RL: MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 26: 5017-5022, 2007.
26. Tazawa H, Tsuchiya N, Izumiya M and Nakagama H: Tumor-suppressive MIR34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci USA* 104: 15472-15477, 2007.
27. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, *et al*: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 17: 1298-1307, 2007.
28. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W and Nikitin AY: microRNA-34b and microRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res* 67: 8433-8438, 2007.
29. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y and Tokino T: Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. *Cancer Res* 68: 4123-4132, 2008.
30. He J, Xu Q, Jing Y, Agani F, Qian X, Carpenter R, Li Q, Wang XR, Peiper SS, Lu Z, *et al*: Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation. *EMBO Rep* 13: 1116-1122, 2012.
31. Lei H, Zou D, Li Z, Luo M, Dong L, Wang B, Yin H, Ma Y, Liu C, Wang F, *et al*: MicroRNA-219-2-3p functions as a tumor suppressor in gastric cancer and is regulated by DNA methylation. *PLoS One* 8: e60369, 2013.
32. Yan X, Chen X, Liang H, Deng T, Chen W, Zhang S, Liu M, Gao X, Liu Y, Zhao C, *et al*: miR-143 and miR-145 synergistically regulate ERBB3 to suppress cell proliferation and invasion in breast cancer. *Mol Cancer* 13: 220, 2014.
33. Hayashi M, Inokuchi M, Takagi Y, Yamada H, Kojima K, Kumagai J, Kawano T and Sugihara K: High expression of HER3 is associated with a decreased survival in gastric cancer. *Clin Cancer Res* 14: 7843-7849, 2008.
34. Begnami MD, Fukuda E, Fregnani JH, Nonogaki S, Montagnini AL, da Costa WL Jr and Soares FA: Prognostic implications of altered human epidermal growth factor receptors (HERs) in gastric carcinomas: HER2 and HER3 are predictors of poor outcome. *J Clin Oncol* 29: 3030-3036, 2011.
35. Oshimo Y, Nakayama H, Ito R, Kitadai Y, Yoshida K, Chayama K and Yasui W: Promoter methylation of cyclin D2 gene in gastric carcinoma. *Int J Oncol* 23: 1663-1670, 2003.
36. Takano Y, Kato Y, Masuda M, Ohshima Y and Okayasu I: Cyclin D2, but not cyclin D1, overexpression closely correlates with gastric cancer progression and prognosis. *J Pathol* 189: 194-200, 1999.
37. Leone V, D'Angelo D, Rubio I, de Freitas PM, Federico A, Colamaio M, Pallante P, Medeiros-Neto G and Fusco A: MiR-1 is a tumor suppressor in thyroid carcinogenesis targeting CCND2, CXCR4, and SDF-1alpha. *J Clin Endocrinol Metab* 96: E1388-E1398, 2011.
38. Zhang L, Liu X, Jin H, Guo X, Xia L, Chen Z, Bai M, Liu J, Shang X, Wu K, *et al*: miR-206 inhibits gastric cancer proliferation in part by repressing cyclinD2. *Cancer Lett* 332: 94-101, 2013.
39. Gong J, Li J, Wang Y, Liu C, Jia H, Jiang C, Wang Y, Luo M, Zhao H, Dong L, *et al*: Characterization of microRNA-29 family expression and investigation of their mechanistic roles in gastric cancer. *Carcinogenesis* 35: 497-506, 2014.