miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer

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Abstract. Growing evidence suggests that microRNAs (miRNAs) are aberrantly expressed in many human cancers, and that they play significant roles in carcinogenesis and cancer progression. The identification of tumor suppressive miRNAs and their target genes could provide new insights into the mechanism of carcinogenesis. However, the genetic or epigenetic regulations of these miRNAs have not yet been fully elucidated in bladder cancer (BC). Chromosomal alterations of cancer cells give us important information for the identification of tumor suppressor genes. Our miRNA array-comparative genomic hybridization (CGH) analysis showed several miRNAs to be candidate tumor suppressors of BC. Our array-CGH analysis revealed that chromosome 4 was lost in all BC cell lines. We selected 19 miRNAs located on chromosome 4 and evaluated their expression levels in cancer cell lines as well as clinical samples. Gain-of-function analysis revealed that miR-218 inhibited BC cell proliferation, migration and invasion. Furthermore, flow cytometry analysis showed that it induced BC cell apoptosis. Genome-wide gene expression analysis showed that it targeted multiple oncogenes in BC. Our study is the first to demonstrate that miR-218 located on chromosomse 4p15.31 is a tumor suppressive miRNA in BC. The identification of tumor suppressive miRNAs and their target genes on the basis of array-CGH analysis could provide new insights into the mechanisms of BC carcinogenesis.

Introduction

Bladder cancer (BC) is the fourth most common cancer in Western industrialized countries and the second most common cancer of the genitourinary tract. In Japan, the age-standardized mortality rate of BC has remained relatively stable in men but has increased slightly since 1993 in women (1). There have been significant advances in treatment, including surgical techniques and adjuvant chemotherapy. However, BC continues to be a common disease with high mortality (2). To date, the mechanisms of BC carcinogenesis have not been fully investigated. Hence, new treatment modalities based on novel molecular targets in BC are desired.

MicroRNAs (miRNAs) are an abundant class of small non-coding RNAs, about 22 nucleotides in length, and they function as negative regulators (cleavage or translational repression) of gene expression by antisense complimentary to specific mRNAs. Although their biological functions remain largely unknown, recent studies have suggested that alterations in miRNA expression contribute to the development of various malignancies. To date, a spectrum of cancer-associated miRNAs has been identified, and some miRNAs function as tumor suppressors and other miRNAs act as oncogenes, inducing or promoting cancer development or progression. We previously determined the miRNA expression signatures specific to BC (3) and reported that several down-regulated miRNAs function as tumor suppressors and that their target genes have typical oncogenic functions (4-6).

Aberrant miRNA expressions in cancers are caused by single nucleotide polymorphism, mutations in pri-miRNA sequences, alterations in the copy number of miRNA coding regions, and so forth (7). Genomic alterations often account for down-regulated miRNA expression in malignancies. For example, the down-regulated miRNAs, the miR-15/miR-16 cluster and miR-213, are located at 13q14 and chromosome 1; these are genomic loss regions in chronic B cell lymphoma and ovarian cancer (8,9). However, the up-regulated miRNAs, let-7a-3 and the miR-9-1 cluster, are respectively found in the genomic gain region of chromosome 22 and 1 in ovarian cancer (9), and the miR-17-92 cluster is located at the gain...
region of 13q31.3 in lung cancer (10). These candidate miRNAs were selected according to the genomic loss and gain regions where they were harbored on the basis of comparative genomic hybridization (CGH) analyses, leading to an important strategy for indentifying tumor-associated miRNAs. However, the genetic or epigenetic regulations of these miRNAs have not yet been fully elucidated in BC. Microarray-based CGH has been used in a number of studies to study copy number instability and aberration type in BC samples. Ours and other studies have shown that frequent copy number gains occur at chromosome 1, 3, 5, 6, 8, 10, 11, 12, 17, 19 and 20, while copy number losses occur at chromosome 2, 4, 5, 8, 9, 10, 11, 13 and 18 in BC (11-15). We focused on chromosome 4 because almost the whole genomic region of chromosome 4 was detected as lost loci in all BC cell lines, and miRNAs on this chromosome were the most numerous of the lost loci examined in our previous study (16). We therefore hypothesized that the chromosomal loss region harbors down-regulated miRNAs that have a tumor suppressive function through the negative regulation of oncogenes in BC. To test this hypothesis, we measured the expression levels of 19 miRNAs located on chromosome 4 in clinical BC samples and BC cell lines. We performed a cell viability assay and apoptosis assay on BC cell lines transfected with candidate miRNAs. Furthermore, we performed an oligo-microarray analysis to identify genes regulated by the candidate miRNAs.

Materials and methods

**BC cell lines and cell culture.** We used four human BC cell lines. T24 and UMUC were obtained from the American Type Culture Collection. KK47, which was established at Kanazawa University from an Asian male patient aged 50 years with a diagnosis of stage I BC, was kindly provided to us by colleagues there, and BOY was established in our laboratory from an Asian male patient aged 66 years diagnosed with stage III BC with lung metastasis (17). These cell lines were maintained in a minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

**Tissue samples.** Tissue samples were taken from 16 BC patients who had undergone cystectomy or transurethral resection of BCs and six normal bladder epithelium (NBE) samples were derived from organ-confined prostate cancer patients who underwent prostatectomy at Kagoshima University Hospital between 2006 and 2009 (Table I). Another series of 28 BCs and 10 NBEs was subjected to real-time RT-PCR for evaluating mRNA expression levels of the target gene in more clinical samples (Table II). The samples were staged in accordance with the tumor-node-metastasis classification system of the American Joint Committee on Cancer/Union Internationale Contre le Cancer (UICC) and were histologically graded (18). Also studied were six NBEs samples derived from organ-confined prostate cancer patients who underwent prostatectomy. The study was approved by the Bioethics Committee of Kagoshima University. Written informed consent and approval were given by the patients prior to the study.

### Table I. Patient characteristics.

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**Tissue collection and RNA extraction.** Tissue samples were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at -20°C until the RNA extraction. Total RNA including miRNA was extracted from frozen fresh tissues using the mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions. The integrity of the RNA was checked with an RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer™ (Agilent Technologies, Santa Clara, CA, USA).

**Quantitative real-time RT-PCR.** TaqMan probes and primers for thioredoxin-related transmembrane protein 1 (TMX1) (TaqMan® Gene Expression Assays; P/N: Hs00991569_m1 for TMX1; Applied Biosystems, Foster City, CA, USA) were used to quantify mRNA expression levels of the target gene in more clinical samples (Table II). The samples were staged in accordance with the tumor-node-metastasis classification system of the American Joint Committee on Cancer/Union Internationale Contre le Cancer (UICC) and were histologically graded (18). Also studied were six NBEs samples derived from organ-confined prostate cancer patients who underwent prostatectomy. The study was approved by the Bioethics Committee of Kagoshima University. Written informed consent and approval were given by the patients prior to the study.
Cell proliferation, wound healing and matrigel invasion assay. Cell proliferation was determined by using an XTT assay (Roche Applied Sciences, Tokyo, Japan) performed according to the manufacturer's instructions. Cell migration activity was evaluated by wound healing assay. Cells were plated in 6-well dishes, and the cell monolayer was scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from photomicrographs. A cell invasion assay was carried out using modified Boyden chambers consisting of transwell-pre-coated matrigel membrane filter inserts with 8-µm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). MEM containing 10% fetal bovine serum in the lower chamber served as the chemoattractant, as described previously (4). All experiments were performed in triplicate.

Apoptosis analysis. BC cell lines transiently transfected with miR-control and miR-218 in 6-well tissue culture plates, as described previously, were harvested 72 h after transfection by trypanosim and washed in cold PBS. Double staining with FITC-Annexin V and propidium iodine (PI) was carried out using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's recommendations and immediately analyzed within an hour by flow cytometry (FACScan®; BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells and apoptotic cells using CellQuest software (BD Biosciences), and then the percentages of early apoptotic cells from each experiment were compared. Experiments were done in triplicate.

Oligonucleotide microarray analysis of BC cell lines. Oligo-microarray Human 44K (Agilent) was used for expression profiling in miR-218-transfected BC cell lines (BOY and T24) in comparison to the miR-negative control transfectant, as previously described (4). Briefly, hybridization and washing steps were performed in accordance with the manufacturer's instructions. The arrays were scanned using a Packard GSI Lumonics ScanArray® 4000 (Perkin-Elmer, Boston, MA, USA). The data were analyzed by means of DNASIS® array software (Hitachi Software Engineering), which converted the signal intensity for each spot into text format. The log2 ratios of the median subtracted background intensity were analyzed. Data from each microarray study were normalized by global normalization.

Statistical analysis and predicting target genes of candidate miRNAs. The relationship between two variables and the numerical values obtained by real-time RT-PCR was analyzed using the Mann-Whitney U test. The relationship between three variables and the numerical values was analyzed using the Bonferroni-adjusted Mann-Whitney U test. Expert StatView® analysis software (version 4, SAS Institute Inc., Cary, NC, USA) was used. In the comparison of three variables, a non-adjusted statistical level of significance of P<0.05 corresponded to a Bonferroni-adjusted level of P<0.0167. We used the TargetScan program (release 5.1, http://www.targetscan.org/) to identify the predicted target genes and their conserved sites for the candidate miRNAs.

Results

Selection of miRNAs located on chromosome 4 by array-CGH analysis. To produce a comprehensive survey of genomic aberrations in BC, in our previous study we had analyzed a BC cell line using array-CGH for patterns of chromosomal gains and/or losses [NCBI GEO (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE19714] (16). Predominantly lost loci were observed at chromosomes 4p and 4q in all of the

Table II. Patient characteristics.

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cell lines. The miRNAs located at chromosomes 4p and 4q were the most numerous among the lost loci. Therefore, we focused on the 23 miRNAs located at the loci for further expression analysis (Fig. 1). Predicted mature miRNAs on the loci were checked by referring to the miRBase (release 16.0, http://microrna.sanger.ac.uk/).

Expression levels of 19 miRNAs located on chromosome 4 in BC cell lines and clinical samples. We validated the results from our array-CGH data by subjecting 19 commercially available miRNAs to stem-loop RT-PCR in four BC cell lines (BOY, KK47, T24 and UMUC) and NBEs (n=6) (Fig. 2A). Four miRNAs (miR-574-5p, miR-297, miR-1979 and miR-1305)
were not commercially available at the time of this experiment (Table III). When we looked at miRNAs for which the expression was <0.01-fold in at least two of four BC cell lines, we found that miR-95, miR-218 and miR-578 remained strongly down-regulated miRNAs on chromosome 4 in BC cell lines (Table III and Fig. 2A). To validate these miRNA expressions in clinical samples, we subjected 16 BCs and six NBEs to stem-loop RT-PCR (Table I). We found a significant difference in miR-218 expression between them (0.111±0.014 and 1.000±0.102, respectively, P=0.0004) (Fig. 2B), but no significant differences in miR-95 expression between BCs and NBEs, and no expression of miR-578 was detected in any of the BCs or NBEs. Consequently, we chose miR-218 for the gain-of-function studies. There were no significant correlations between miRNA expression and clinicopathological parameters (data not shown).

Effect of miR-218 transfection on cell proliferation, migration, invasion activity and cell apoptosis in BC cell lines. To examine the functional role of these miRNAs, we performed gain-of-function studies using the miRNA transfectants. The XTT assay showed significant cell proliferation inhibition in miR-218 transfectants compared to the controls from BOY and T24 cell lines (% of cell proliferation; BOY, 55.4±1.6 and 100±4.8, P<0.0001; T24, 72.9±1.6 and 100±1.5, P<0.0001; Fig. 3A). The wound healing assay also demonstrated significant cell migration inhibition in the miR-218 transfectant compared to the counterparts (% of wound closure; BOY, 32±5.3 and 100±8.8, P=0.0001; T24, 51.6±9 and 100±6, P=0.0018; Fig. 3B). The matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in the miR-218 transfectant compared to the counterparts (BOY, 105.6±9 and 238.1±7.9, P=0.0008; T24, 109.3±3.6 and 244.6±13.2, P=0.0008; Fig. 3C). The early apoptotic cell fractions (right lower quadrant) were greater in the miR-218 transfectant than in the miR-control transfectant (early apoptotic cells relative to the control; BOY, 5.96±1.07 and 1.00±0.40, P=0.0016; T24, 3.63±0.35 and 1.00±0.16, P=0.0008) (Fig. 3D). These results suggest that miR-218 expression can induce cell apoptosis in BC cells.

Gene expression profile identifying differentially expressed genes in miR-218 transfectant. To gain further insight into which genes are down-regulated by miR-218 in BC, we performed gene expression analysis of miR-218 transfectants (BOY and T24) (Fig. 4). We identified 162 genes that were generally up-regulated in miR-218 transfectants by >2-fold compared to the transfectant. In contrast, 337 genes...
were down-regulated by <2-fold in the transfectants. The functional annotations of the up- and down-regulated genes were respectively classified into 11 and 8 categories (Fig. 4). The gene expression profile of miR-218 transfectants demonstrated that the up-regulated gene categories included tumor suppressive categories, such as regulation of transcription, anatomical structure development and apoptosis, whereas down-regulated gene categories included oncogenic categories, such as signal transduction, regulation of apoptosis and cell cycle. The oligonucleotide array data are available for reference.

Figure 3. Gain of function studies in BC cell lines with miR-218 transfectants. (A) Cell proliferation determined by the XTT assay in BOY and T24 cell lines transfected with the miRNAs. **P<0.0001. (B) Significant cell migration inhibitions were observed in BOY and T24 cell lines transfected with miR-218. Phase micrographs of BOY and T24 cell lines taken at 0 and 24 h after monolayer wounding are shown on the left panel. Quantification of cell migration using the monolayer wound healing assay is shown on the right panel. *P<0.01. (C) Significant cell invasion inhibitions were observed in BOY and T24 cell lines transfected with miR-218. Phase micrographs of invading BOY and T24 cell lines are shown on the left panel. Quantifications of cell invasion are shown on the right panel. *P<0.0001. (D) Apoptosis assay by flow cytometry. Significant early apoptotic cells were observed in BOY and T24 cell lines transfected with miR-218. Early apoptotic cells can be seen in the bottom right quadrant (D, left panel). The normalized ratio of the apoptosis assay is shown in the histogram. Percentage of early apoptotic cells out of the total measured cell population for miR-control and miR-218-transfected BC cell lines. *P<0.01. **P<0.0001.
Identification of candidate miR-218 target genes by expression profiles. On the basis of our oligo-microarray data, TMX1 was at the top of the down-regulated genes that had conserved sites for miR-218. Therefore, we focused on this gene as a target of miR-218. The quantitative real-time RT-PCR analysis showed that the mRNA expression levels of TMX1 in the BOY and T24 cell lines were higher than in the NBEs (n=10) (Fig. 5A), and the expression levels of TMX1 were markedly repressed in the miR-218 transfectants in comparison to the control (Fig. 5B). Our quantitative real-time RT-PCR showed that there was a trend but no significant difference in TMX1 mRNA expression between clinical BCs and NBEs (p=0.0734) (Fig. 5C). There were no significant correlations between TMX1 mRNA expression and clinicopathological parameters (data not shown).

Discussion

The mechanisms underlying miRNA gene expression in cancer are not well understood. Although genomic alterations are critical in oncogenesis (19,20), studies have so far focused mostly on protein-coding genes. As more than half of the miRNAs have been aligned to genomic fragile sites or regions associated with cancers, miRNA expression levels could change along with the gene copy number of where they were harbored. Calin et al showed that the miR-15/miR-16 cluster was located at chromosome 13q14, a region deleted in more than half of chronic B cell lymphomas. They focused on the miR-15/miR-16 cluster located within a 30-kb region on the basis of CGH analysis of chronic B cell lymphoma (8). Previous studies have reported copy number losses in chromosomes 4p and 4q in clinical BC and BC cell lines (21-24). Consistent with these previous studies, our array-CGH data demonstrated that chromosome 4 was the typical chromosomal loss region and miRNAs on this chromosome were the most numerous of the lost loci, suggesting that inactivating these miRNAs on chromosome 4 could play an important role in BC development. A recent study identified that miR-218, which is located at chromosome 4p15.31, was down-regulated according to genome-wide array-CGH data combined with a bioinformatics
mapping approach for lung cancer (25). Consistently, we found that the expression levels of certain miRNAs, including miR-218 were down-regulated in clinical BC specimens as well as BC cell lines. Thus, miRNA profiling based on our array-CGH data is a new strategy for finding crucial miRNAs in human BC. However, there was no typical down-regulation of several miRNAs on chromosome 4 in this study. Some miRNAs, such as miR-548i-1, -2, -3 and -4, are located at several chromosomal loci and have common mature miRNA sequences but are located at different chromosomes. Hence, their expression levels might not decrease unless all loci are simultaneously deactivated. In terms of miR-218, there are two genes that code for mature miR-218, miR-218-1 is located on chromosome 4p15.31, and miR-218-2 is located on chromosome 5p34. We observed a genomic loss region on chromosome 4p15.31, but not on chromosome 5p34 in the same region of chromosome 4p15, implying that large losses in this region might be crucial for BC carcinogenesis and development. Another study demonstrated that prosurvival protein 1 (VOPP1), which activated nuclear factor κB (NF-κB) and was associated with anti-apoptotic response, was a direct target of miR-218 (26). ROBO1 and VOPP1 were among the top 20 down-regulated genes in our oligo-microarray data using miR-218-transfected BC cell lines (Table IV), suggesting that these molecules may have tumor suppressive functions and could be directly regulated by miR-218 in BC.

The gene expression profile of miR-218 transfectants in this study suggests that miR-218 expression can affect down-regulating genes classified into oncogenic categories and up-regulating genes classified into tumor suppressive categories. The expression levels of the TMX1 gene, which was the top down-regulated gene in miR-218 transfectants and was reported to have an anti-apoptotic function (35), was actually overexpressed in clinical BC samples and down-regulated by miR-218 transfection. These results suggest that miR-218 and its target gene may be a gene therapy candidate for clinical BC. In this study, there was no significant relation-

### Table IV. Top 20 down-regulated genes, which have putative miR-218 target sites, in miR-218 transfectants.

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</tbody>
</table>

miR-218-1 is located within the intron 15 of slit homologue 2 (SLIT2), a tumor suppressor gene. SLIT2 has been intensively investigated, and it has been found to be frequently down-regulated in lung cancers, breast cancers (31), glioma (32), cervical cancer (33) and hepatocellular carcinoma (34). However, miR-218 directly regulates roundabout 1 (ROBO1), which is known to be a receptor of SLIT2 (SLIT/ROBO pathway) by inducing apoptosis in certain human malignancies. Simultaneous inactivations of miR-218 and SLIT2, which are in the same region of chromosome 4p15, imply that large losses in this region might be crucial for BC carcinogenesis and development. Another study demonstrated that prosurvival protein 1 (VOPP1), which activated nuclear factor κB (NF-κB) and was associated with anti-apoptotic response, was a direct target of miR-218 (26). ROBO1 and VOPP1 were among the top 20 down-regulated genes in our oligo-microarray data using miR-218-transfected BC cell lines (Table IV), suggesting that these molecules may have tumor suppressive functions and could be directly regulated by miR-218 in BC.
ship between miR-218 expression and clinicopathological parameters. Our cohort was too small to evaluate the relationship between them.

In conclusion, we demonstrate that miR-218 is down-regulated in BC and that it is located in the loss locus, 4p15.31, in BC cell lines. We found decreased cell proliferation, migration and invasion activity, and increased cell apoptosis in miR-218 transfectants, suggesting that miR-218 is a candidate tumor suppressive miRNA of human BC.

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