Dual tumor-suppressors miR-139-5p and miR-139-3p targeting matrix metalloprotease 11 in bladder cancer

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Key words
Bladder cancer, miR-139, miRNA, MMP11, tumour suppressors

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Funding Information
JSPS KAKENHI Grant (Grant/Award Numbers: 16K11015, 16K20415, 16K15691, 26293354 and 26462416)

Received April 5, 2016; Revised June 21, 2016; Accepted June 29, 2016
Cancer Sci 107 (2016) 1233–1242
doi: 10.1111/cas.13002

Bladder cancer (BC) is the most common cancer of the urinary tract, and the ninth most common cause of death worldwide. In the USA alone, it was estimated that 76,000 new cases were diagnosed and 1600 patients died in 2015.1 BC can be categorized into two groups: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Approximately 70–80% of patients are diagnosed with NMIBC, and some of them have a high risk of recurrence and a variable risk of progression despite local therapies.2 The remaining 25% of MIBC patients are managed by radical surgery or radiotherapy, but often still have poor outcomes despite systemic therapies.3-5 For patients with advanced BC or failure of first-line treatment, there are no clearly defined second-line treatments and none have definitively been shown to prolong overall survival.4 Therefore, elucidation of novel molecular mechanisms of recurrence and muscle invasion are urgent issues associated with this disease.

MicroRNA (miRNA) are endogenous small non-coding RNA molecules (19–22 bases in length) that regulate the expression of protein-coding/protein non-coding genes in a sequence-specific manner.6,7 A substantial amount of evidence has demonstrated that aberrantly expressed miRNA can act as oncoprogens or tumor suppressors. These miRNA can disrupt tightly controlled RNA networks in cancer cells.8 Identification of aberrantly expressed miRNA in cancer cells could provide important clues for the investigation of novel molecular mechanisms of initiation, progression and metastasis in cancer cells.

In miRNA biogenesis, it is the general consensus that processing of the pre-miRNA through Dicer1 generates a miRNA duplex (a passenger strand and a guide strand), and that the passenger strand has no regulatory activity and disintegrates in cells.9 More recently, we showed that miR-144-5p (passenger strand) and miR-144-3p (guide strand) induced cell cycle arrest and acted as tumor suppressors in BC cells. Moreover, miR-144-5p directly regulated several cell cycle related genes, including CCNE1, CCNE2, CDC25A and PKMYT1.8 Our data strongly suggested that both miRNA were downregulated in BC tissues. The aim of this study was to investigate the functional roles of these miRNA and their modulation of cancer networks in BC cells. Functional assays of BC cells were performed using transfection of mature miRNA or small interfering RNA (siRNA). Genome-wide gene expression analysis, in silico analysis and dual-luciferase reporter assays were applied to identify miRNA targets. The associations between the expression of miRNA and its targets and overall survival were estimated by the Kaplan–Meier method. Gain-of-function studies showed that miR-139-5p and miR-139-3p significantly inhibited cell migration and invasion by BC cells. The matrix metalloprotease 11 gene (MMP11) was identified as a direct target of miR-139-5p and miR-139-3p. Kaplan–Meier survival curves showed that higher expression of MMP11 predicted shorter survival of BC patients (P = 0.029). Downregulated miR-139-5p or miR-139-3p enhanced BC cell migration and invasion in BC cells. MMP11 was directly regulated by these miRNA and might be a good prognostic marker for survival of BC patients.
in BC cells, and will facilitate the development of novel diagnostic and therapeutic strategies for treatments of the disease.

Materials and Methods

Clinical specimens and cell culture. The tissue specimens were collected from 62 BC patients at Kagoshima University Hospital between 2003 and 2013. Normal bladder epithelia (NBE) \((n = 23)\) were derived from patients with noncancerous disease. The specimens were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer (UICC) TNM classification and histologically graded.\(^9\)

Our study was approved by the Bioethics Committee of Kagoshima University; prior written informed consent and approval were obtained from all patients. Clinicopathological characteristics of the patients are listed in Table S1. We used two human BC cell lines: T24 and BOY. These cell lines were described in our previous studies.\(^10–12\)

Tissue collection and RNA extraction. Tissues were immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and stored at \(-20^\circ\)C until RNA extraction was conducted. Total RNA, including miRNA, was extracted using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) following the manufacturer’s protocol.

Quantitative real-time RT-PCR. Stem-loop RT-PCR (TaqMan MicroRNA Assays; product ID: 17100 for miR-139-5p and product ID: 17100 for miR-139-3p; Applied Biosystems, Foster City, CA, USA) was used to quantify miRNA according to the manufacturer’s protocol for PCR conditions. TaqMan probes and primers for Matrix metalloprotease 11: MMP11 (product ID: Hs 00968295_m1; Applied Biosystems) were assay-on-demand gene expression products. We used human GUSB
molecules. We used pre-miR miRNA precursors (AM17111; Thermo Fisher Scientific), PM25489, and negative control miRNA; product ID: Lipofectamine RNAiMAX transfection reagent and Opti-MEM and negative control siRNA (product ID: D-001810-10; Thermo Fisher Scientific).

Mature miRNA and small interfering RNA transfection. As described previously,(10–12) BC cell lines were transfected with Lipofectamine RNAiMAX transfection reagent and Opti-MEM (Thermo Fisher Scientific) with 10–30 nM mature miRNA molecules. We used pre-miR miRNA precursors (hsa-miR-139-5p; product ID: PM11749, hsa-miR-139-3p; product ID: PM25489, and negative control miRNA; product ID: AM17111; Thermo Fisher Scientific), si-MMP11 (product ID: HSS105529 and HSS179967; Thermo Fisher Scientific) and negative control siRNA (product ID: D-001810-10; Thermo Fisher Scientific).

Cell proliferation, migration and invasion assays. Cell proliferation, migration and invasion assays were carried out as previously described.(10–12) 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 split into six-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-mm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). MEM containing 10% FBS in the lower chamber served as the chemoattractant. All experiments were performed in triplicate.

**Western blot analyses.** After transfection (72 h), protein lysates were separated on NuPAGE 4–12% Bis-Tris gels (Thermo Fisher Scientific) and transferred onto PVDF membranes. Immunoblotting was conducted with diluted monoclonal anti-MMP11 antibodies (1:250, ab52904; Abcam, Cambridge Science Park in Cambridge, UK) and with diluted anti-GAPDH antibodies (1:5000, MAB374; Chemicon, Temecula, CA, USA). The membrane was washed and then incubated with goat anti-rabbit or mouse IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized with an echochemiluminescence (ECL) detection system (GE Health-care, Little Chalfont, UK).

**Putative target gene analysis of miR-139-5p/miR-139-3p and MMP11.** To investigate the expression status of candidate miR-139-5p/miR-139-3p target genes in BC clinical specimens, we examined gene expression profiles in the Gene Expression Omnibus (GEO) database (accession number: GSE11783+GSE31684). A SurePrint G3 Human GE 8×60K Microarray (Agilent Technologies, Santa Clara, CA, USA) was adopted for expression profiling of miR-139-5p/miR-139-3p and si-MMP11 transfectants. We merged these datasets and selected putative miR-139-5p and miR139-3p target genes using microRNA.org (August 2010 release, http://www.microrna.org).(13) The strategies for investigation of the target genes are shown in Figures S1 and S2.

**Plasmid construction and dual-luciferase reporter assay.** Partial wild-type sequences of the 3' untranslated region (UTR) of MMP11 or those with a deleted miR-139-5p or miR-139-3p target site were inserted between the XhoI and Pmel restriction sites in the 3' UTR of hRLuc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure
for dual-luciferase reporter assay has been described previously.\(^{[10–12,14]}\)

**Immunohistochemistry in tissue microarray.** A tissue microarray of bladder cancer samples was obtained from Biomax (BL1002; Rockville, MD, USA). Detailed information on all tumor specimens can be found at www.biomax.us/index.php. Patient characteristics are summarized in Table S2. The tissue microarray was immunostained following the manufacturer’s protocol with an UltraVision Detection System (Thermo Scientific). The primary rabbit monoclonal antibodies against MMPI1 (ab52904; Abcam) were diluted 1:100. The immunostaining result was evaluated using the scoring method described previously.\(^{[15]}\) All samples were independently scored by two of the authors (M. Yonemori and R. Matsumita), who were blinded to the patient status.

**Statistical analysis.** Relationships between two or three variables and numerical values were analyzed using the Mann–Whitney U-test or the Bonferroni-adjusted Mann–Whitney U-test. The relationship between miR-139-5p/miR-139-3p and MMPI1 mRNA expression was analyzed with the Spearman rank correlation. We estimated overall survival by using the Kaplan–Meier method. These analyses were carried out by using Expert Stat View software, version 5.0 (Cary, NC, USA).

**Results**

The expression levels of miR-139-5p/miR-139-3p in bladder cancer specimens and bladder cancer cell lines. We evaluated the expression levels of miR-139-5p/miR-139-3p in BC tissues (n = 62), normal bladder epithelia (NBE) (n = 23) and BC cell lines (T24 and BOY). The expression levels of miR-139-5p/miR-139-3p were significantly lower in tumor tissues and BC cell lines than in NBE (P < 0.0001, Fig. 1a upper and middle). There was a positive correlation between the expression levels of miR-139-5p/miR-139-3p (r = 0.925, P < 0.0001, Fig. 1a, bottom). There were no significant correlations between any of the clinicopathological parameters (i.e. tumor stage and grade) and the expression of miR-139-5p or miR-139-3p (data are not shown).

In human genome, pre-miR-139 (miR-139-5p and miR-139-3p) is located on the human chromosome 11q13.4 region and this miRNA is encoded within the second intron of phosphodiesterase 2A (PDE2A) gene (Fig. S3a). We also investigated the expression of PDE2A (host gene of pre-miR-139) and confirmed the downregulation of PDE2A in BC clinical specimens and cell lines (Fig. S3c,d). To investigate the molecular mechanisms of silencing of these miRNA, BC cells were treated with the demethylating agent [5-aza-2′-deoxycytidine (5-aza-dC)]. Expression of miR-139-5p, miR-139-3p and PDE2A were significantly elevated after 5-aza-dC treatment in BC cells (Fig. S3b). These data indicated that DNA methylation might cause silencing of miR-139-5p and miR-139-3p in BC cells.

**Effects of restoring miR-139-5p and miR-139-3p on cell proliferation, migration and invasive activities in bladder cancer cell lines.** To examine the functional roles of miR-139-5p and miR-139-3p, we performed gain-of-function studies by using miRNA-transfected BOY and T24 cells. XTT assays revealed that there was a significant inhibition of cell proliferation in the miR-139-3p-transfectants (P < 0.0001), but not in the miR-139-5p-transfectants (Fig. 1b). Wound healing and Matrigel invasion assays demonstrated significant inhibition of cell migration and invasion in both the miR-139-5p and
miR-139-3p-transfectants (P < 0.0001 or P < 0.005) (Figs 1c, d, and S4). To investigate the synergistic effects of miR-139-5p and miR-139-3p, we performed the functional assays (cell proliferation, migration and invasion) by co-transfection of mature miR-139-5p and miR-139-3p into the bladder cancer cell lines (T24 and BOY). However, we found no synergistic effect by co-transfection of miR-139-5p and miR-139-3p (Fig. S5).

Screening of putative genes commonly targeted by miR-139-5p and miR-139-3p in bladder cancer. To gain further insight into the molecular mechanisms regulated by tumor-suppressive miR-139-5p/miR-139-3p in BC cells, we screened miR-139-5p
and miR-139-3p-regulated genes by using in silico and genome-wide gene expression analysis. First, we examined gene expression signatures that were upregulated in clinical BC specimens from GEO database and found that 9134 genes were significantly upregulated in 90 BC compared to 6 NBE. Importantly, we found that in T24 521 of those genes were downregulated in miR-139-5p-transfectants. Moreover, 1304 genes were downregulated in miR-139-3p transfectants of T24. We narrowed the number of target genes down to 179 and 277 as putative targets for miR-139-5p or miR-139-3p, respectively, by means of the miRNA.org database. Finally, a total of 22 genes were listed as promising candidates that were commonly targeted by both miRNA in BC (Table 1). Then, we focused on the MMP11 gene because it was at the top of the list.

**MMP11 was directly regulated by miR-139-5p and miR-139-3p.** We performed quantitative real-time RT-PCR to determine whether restoration of miR-139-5p or miR-139-3p resulted in downregulating MMP11 mRNA expression in T24 and BOY cells. The mRNA levels of MMP11 were significantly reduced in miR-139-5p or miR-139-3p transfectants in comparison with mock or miR-control transfectants (P < 0.001, Fig. 2a). The protein expression levels of MMP11 were also significantly reduced in the transfectants (Fig. 2b). Furthermore, we performed dual-luciferase reporter assays in BC cells to determine whether the gene was directly regulated by miR-139-5p or miR-139-3p. The microRNA.org database predicted that there was a binding site for miR-139-5p and one for miR-139-3p in the 3′-UTR of MMP11 (positions 101–117 and 87–108, respectively) (Fig. 2c). We used vectors encoding the partial wild-type sequence of the 3′-UTR of the mRNA, including the predicted miR-139-5p or miR-139-3p target sites. We found that the luminescence intensity was significantly reduced by co-transfection with miR-139-5p or miR-139-3p and the vector carrying the wild-type 3′-UTR, whereas transfection with the deletion vector (the binding site had been removed) blocked the decrease in luminescence (P < 0.001, Fig. 2d). These data suggested that miR-139-5p and miR-139-3p bound directly to specific sites in the 3′-UTR of MMP11.

**Effects of MMP11 knockdown on cell proliferation, migration and invasion in bladder cancer cells.** To investigate the functional role of MMP11, loss-of-function studies were carried out by examining si-MMP11 transfectants. First, the knockdown efficiency of si-MMP11 transfection was evaluated in BOY and T24 cells. Western blot analyses and quantitative real-time RT-PCR showed that si-MMP11 effectively downregulated MMP11 expression in T24 and BOY cells (Fig. 3a,b). Wound healing and Matrigel invasion assays showed that cell proliferation, migration and invasion were inhibited in si-MMP11 transfectants compared to mock or si-control transfectants in T24 and BOY cells (Fig. 3c–e).

**Expression of MMP11 in bladder cancer clinical specimens.** Quantitative real-time RT-PCR analyses showed that expression levels of MMP11 were significantly upregulated in 62 BC specimens compared with 23 NBE (P = 0.0061, Fig. 4a). Spearman’s rank test showed negative correlation between miR-139-5p/miR-139-3p expression and MMP11...
mRNA expression ($r = -0.21$ and $-0.25$, $P = 0.058$ and $P = 0.022$, Fig. 4b). Next, we evaluated the correlation of MMP11 expressions with clinicopathological factors (Fig. 5). The expression level of MMP11 was significantly higher in BC with lymph node metastasis (N1 ≤) ($P = 0.013$) and distant metastasis (M1) ($P = 0.022$, Fig. 5). We examined the correlation of MMP11 expression levels with prognosis using the Kaplan–Meier method. Kaplan–Meier analysis showed that the high MMP11 expression group had significantly lower overall survival (OS) probabilities compared to the low MMP11 expression group ($P = 0.029$, Fig. 6). However, Cox proportional hazards regression multivariate analysis revealed that the expression level of MMP11 was not an independent predictor of OS (Table S3).

**Immunohistochemistry in tissue microarray.** Representative immunohistochemistry of MMP11 is shown in Figure 7. The MMP11 was strongly expressed in BC compared with NBE. The expression score of BC was significantly higher than that of NBE ($P = 0.0186$, Fig. 7a). However, we found no significant difference between the MMP11 expression score and pathological parameters (tumor stage and grade) of BC patients (Table S4).

**Downstream gene regulated by miR-139-5p/miR-139-3p/MMP11 axis.** To identify the downstream genes regulated by the miR-139-5p/miR-139-3p/MMP11 axis, a genome-wide gene expression analysis and *in silico* analysis were performed in two BC cell lines transfected with si-MMP11. Following the strategy of Figure S2, we identified a total of 116 genes. Some portion of them may have oncogenic function through miR-139-5p/miR-139-3p/MMP11 axis (Tables 2 and S5).

To validate the putative candidate of downstream genes which regulated by miR-139-5p/miR-139-3p/MMP11 axis in BC cells, we investigated the expression levels of SPC24, CYR61, CXCL1, CXCL3 and IL6 in miR-139-5p, miR-139-3p and si-MMP11 transfectants by RT-PCR (Table S6). Expression levels of these genes were reduced by miR-139-5p, miR-139-3p and MMP11 transfections, suggesting these genes were regulated by miR-139-5p/miR-139-3p/MMP11 axis in BC cells (Fig. S6).

**Discussion**

Aberrantly expressed miRNA can disturb normally regulated RNA networks and disrupt physiologic processes in cancer cells. Strategies to identify aberrant expression of miRNA-mediated cancer pathways are being developed, and they represent a new direction in cancer research in the post-genome era.

![Fig. 6.](image) The association between the expression level of MMP11 and overall survival. Kaplan–Meier survival curves for overall survival rate based on MMP11 expression in 62 BC patients. *P*-values were calculated using the log-rank test.

![Fig. 7.](image) Immunohistochemical staining of MMP11 in tissue specimens. (a) There was a significant difference in the expression score of MMP11 of 80 BCs in comparison with 20 NBEs. (b) Immunohistochemical staining of MMP11 in tissue specimens (magnification ×40 and ×200). Immunoreactivity for MMP11 was obtained in the cytoplasm of the BC cells and in the fibrous stroma (Right and middle). Normal bladder epithelia were completely negative or faintly positive (left).
Table 2. Top 20 genes of significantly enriched annotations (downregulated genes of miR-139 and si-MMP11 transfectants)

| Gene ID  | Gene symbol | Description | GEO Fold change | T2A si-MMP11-2 | BOY si-MMP11-2 | T2A miR-139-5p | T2A miR-139-3p |
|----------|-------------|-------------|----------------|----------------|----------------|----------------|----------------|
| 147841   | SPC24       | SPC24, NDC80 kinesin complex component | 15.185 | 4.98E-05 | -1.356 | -1.650 | -0.992 | -0.674 |
| 4320     | MMP11       | Matrix metalloproteinase 11 (stromelysin 3) | 15.126 | 4.38E-05 | -2.153 | -2.006 | -0.771 | -0.950 |
| 389247   | LOC389247   | Uncharacterized LOC389247 | 13.938 | 5.32E-05 | -0.525 | -0.591 | -0.282 | -1.616 |
| 3491     | CYR61       | Cysteine-rich, angiogenic inducer, 61 | 12.826 | 5.67E-05 | -1.684 | -1.828 | -0.102 | -0.266 |
| 2919     | CXCL1       | Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) | 12.821 | 7.32E-05 | -0.853 | -0.507 | -1.526 | -1.639 |
| 2921     | CXCL3       | Chemokine (C-X-C motif) ligand 3 | 12.257 | 1.21E-04 | -1.006 | -1.010 | -1.127 | -0.615 |
| 51514    | DTL         | Denticlineless E3 ubiquitin protein ligase homolog (Drosophila) | 11.071 | 4.38E-05 | -0.798 | -0.951 | -0.306 | -1.656 |
| 11004    | KIF2C       | Kinesin family member 2C | 10.824 | 4.38E-05 | -0.593 | -0.887 | -0.276 | -0.909 |
| 113730   | KLHDC7B     | Kelch domain containing 7B | 9.112 | 4.98E-05 | -1.731 | -0.758 | -0.672 | -0.893 |
| 9156     | EXO1        | Exonuclease 1 | 8.409 | 4.38E-05 | -0.657 | -0.652 | -0.159 | -0.712 |
| 3569     | IL6         | Interleukin 6 | 7.617 | 4.38E-05 | -1.009 | -0.563 | -1.160 | -2.630 |
| 150051   | LOC150051   | Uncharacterized LOC150051 | 6.789 | 1.00E-04 | -1.513 | -0.722 | -0.088 | -0.751 |
| 51659    | GINS2       | GINS complex subunit 2 (Psf2 homolog) | 6.727 | 4.67E-05 | -0.818 | -1.367 | -0.003 | -1.231 |
| 4837     | NNMT        | Nicotinamide N-methyltransferase | 6.119 | 6.25E-04 | -2.733 | -2.213 | -0.420 | -1.782 |
| 7015     | TERT        | Telomerase reverse transcriptase | 5.801 | 7.32E-05 | -0.874 | -0.945 | -0.621 | -0.849 |
| 7130     | TNFAIP6     | Tumor necrosis factor, alpha-induced protein 6 | 5.781 | 6.60E-04 | -0.645 | -2.109 | -1.119 | -1.682 |
| 147700   | KLK3        | Kinesin light chain 3 | 5.407 | 1.74E-04 | -1.095 | -0.573 | -0.741 | -0.325 |
| 9454     | HOMER3      | Homer homolog 3 (Drosophila) | 5.143 | 4.98E-05 | -0.611 | -0.511 | -0.577 | -0.664 |
| 25886    | POClA       | POCl1 centriolar protein A | 5.113 | 4.38E-05 | -0.530 | -0.558 | -0.139 | -1.259 |
| 1869     | EZF1        | EZF transcription factor 1 | 5.036 | 4.67E-05 | -0.730 | -1.332 | -0.118 | -1.049 |

The sequencing era. Based on the miRNA expression signature of BC cells, we have continued the identification of novel tumor-suppressive miRNA and their dysregulated BC oncogenic targets and pathways. Our past studies demonstrated that clustered miRNA, miR-1/133a, miR-195/497 and miR-23b/27b/24-1 had tumor-suppressive functions through their targeting of several oncogenic genes and pathways in BC cells. (8)–(10,12–15)

Our deep sequencing-based miRNA signature analysis of BC showed that miR-144-5p and miR-144-3p (derived from pre-miR-144) function as anti-tumor effectors in BC cells. (8) More recently, we demonstrated that both strands of pre-miR-145, miR-145-5p (guide-strand) and miR-145-3p (passenger-strand), act as antitumor miRNA in BC cells by regulating UHRF1. (16) The passenger strand has been considered to be immediately degraded after generation and have no functional role. Unlike the past concept, our data indicated that the passenger strand of miRNA has actually functioned in cancer cells. These study showed that both guide and passenger strands of miRNA have biological functions through their regulation of several genes in BC cells. These findings overturn past concepts of miRNA biogenesis. In this study, we focused on the miR-139-5p (guide strand) and miR-139-3p (passenger strand) because these miRNAs were significantly reduced in BC cells based on data from deep sequencing-based miRNA signatures. (10) Our present data showed that restoration of miR-139-5p or miR-139-3p significantly inhibited cancer cell migration and invasion, suggesting that both strands of miR-139 had anti-tumor effects in BC cells. We found no synergistic effect by co-transfection of miR-139-5p and miR-139-3p. In contrast, cell viabilities measured by cell proliferation, migration and invasion were relatively restored in the co-transfectants compared to the independent transfectants (miR-139-5p or miR-139-3p) (Fig. 5). Because of the complementary binding between miR-139-5p and miR-139-3p, each miRNA could not function enough in the co-transfection experiments. Therefore, antitumor effects might decrease in the co-transfection experiments compared to the independent transfection of each miRNA.

Recent studies indicated that miR-139-5p had anti-tumor effects in several types of cancer. (17,18) Ectopic expression of miR-139-5p in non-small cell lung cancer cell lines suppressed cell growth, metastasis and induction of apoptosis through inhibition of CCND1, KIP27, MMP7, MMP9 and c-MET. (18) In hepatocellular carcinoma (HCC), miR-139-5p regulates ROCK2 in cancer cell, and its reduction of miR-139-5p promoted the metastatic abilities of HCC cells. (17) These previous studies and our present analysis provide multiple examples that reveal anti-cancer effects of miR-139-5p in different type cancer cells. In contrast to miR-139-5p, the function of miR-139-3p in cancer cells is still ambiguous. It is important to investigate the functional significance of miR-139-3p and its target genes in the various types of cancer.

Bioinformatic predictions indicate that more than 60% of transcripts in the human genome are regulated by miRNA. (19) Downregulated tumor-suppressive miRNA permit expression of oncogenes. We speculate that miR-139-5p and miR-139-3p work in concert to downregulate genes that promote metastasis in BC cells. To better understand BC metastasis, we identified common target genes of miR-139-5p and miR-139-3p by use of in silico analysis. Molecular target searches revealed that MMP11 was directly regulated by miR-139-5p and miR-139-3p in BC cells. Overexpression of MMP11 was confirmed in BC clinical specimens. Furthermore, Kaplan-Meier analysis showed that high MMP11 expression groups had significantly lower overall survival. Thus, MMP11 has the potential to be a good prognostic marker and therapeutic target in BC.

MMP11 (initially named Stromelysin-3) was cloned in 1990 from a cDNA library constructed from breast cancer surgical specimens. (20) Matrix metalloproteinases (MMP) comprise a
super-family of zinc-dependent endopeptidases, and numerous studies have demonstrated that MMP can enhance cancer cell migration, invasion and metastasis through degradation of the extracellular matrix (ECM). Unlike other MMP, MMP11 does not cleave any classical ECM components, suggesting that it plays indirect roles in ECM remodeling in cancer cells. Previous studies showed that high expression of MMP11 correlated with poor prognosis and predicted recurrence in breast cancer. A recent study of colorectal cancer showed that MMP11 expression was a predictive survival marker of colorectal cancer patients. Moreover, oncogenic roles of MMP11 were reported in several cancers, including cervical cancer and gastric cancer. Our present data indicated that MMP11 expression was significantly related to survival of BC patients, suggesting MMP11 and its regulated pathways might be a potential target of treatment of this disease. More detailed studies of the function of MMP11 in BC cells are necessary. Current data have shown that the expression of MMP affected multiple signaling pathways that modulate both normal and pathologic processes.

To investigate the molecular mechanisms of MMP11 in BC cells, we applied a genome-wide gene expression analysis using si-MMP11 transfectants. Our data showed that several genes known to contribute to cancer cell aggressiveness were downstream from MMP11, such as CXCL1 and CXCL3. Aberrant expression and activation of chemokines are known to enhance malignancies of several cancers. CXCL1 interacts with the G-proteincoupled receptor CXCR2, and plays a key role in inflammation. Overexpressed CXCL1 has been reported in colon, skin, renal, prostate and breast cancers. Past studies showed that CXCL1 modulates the invasive abilities of BC cells and this chemokine may be a potential biomarker for invasive bladder cancer. Expression of CXCL1 is upregulated in more aggressive phenotype of BC and high expression of CXCL1 has been associated with reduced disease-free survival.

A recent report demonstrated that CXCL3 and its receptor CXCR2 were overexpressed in prostate cancer cells and expression levels of CXCL3 were closely related to metastatic prostate cancer. Moreover, CXCL3 is involved in the metastasis of breast cancer. These findings suggested that CXCL1 and CXCL3 may be potential targets for cancer therapy, including BC. The identification of novel molecular pathways regulated by the miR-139-5p/miR-139-3p/MMP11 axis may lead to a better understanding of BC progression and metastasis.

In conclusion, downregulation of dual-strand miR-139-5p and miR-139-3p was validated in BC clinical specimens, and these miRNA were shown to function as tumor suppressors in BC cells. To the best of our knowledge, this is the first report demonstrating that tumor-suppressive miR-139-5p and miR-139-3p directly targeted MMP11. Moreover, MMP11 was upregulated in BC clinical specimens and contributed to migration and invasion through its regulation of several oncogenic genes. Expression of MMP11 might be a useful prognostic marker for survival of BC patients. The identification of novel molecular pathways and targets regulated by the miR-139-5p/miR-139-3p/MMP11 axis may lead to a better understanding of BC progression and aggressiveness.

Acknowledgments

This study was supported by JSPS KAKENHI Grant Numbers, 16K11015, 16K20415, 16K15691 and 26426421. We thank Ms M. Miyazaki for excellent laboratory assistance.

Disclosure Statement

The authors have no conflicts of interest to declare.
24 Tian X, Ye C, Yang Y et al. Expression of CD147 and matrix metalloproteinase-11 in colorectal cancer and their relationship to clinicopathological features. J Transl Med 2015; 13: 337.

25 Vazquez-Ortiz G, Pina-Sanchez P, Vazquez K et al. Overexpression of cathepsin F, matrix metalloproteinases 11 and 12 in cervical cancer. BMC Cancer 2005; 5: 68.

26 Deng H, Guo RF, Li WM, Zhao M, Lu YY. Matrix metalloproteinase 11 depletion inhibits cell proliferation in gastric cancer cells. Biochem Biophys Res Commun 2005; 326: 274–81.

27 Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinas: regulators of the tumor microenvironment. Cell 2010; 141: 52–67.

28 Hembrough SL, Cheng N. Chemokine signaling in cancer: implications on the tumor microenvironment and therapeutic targeting. Cancer Therapy 2009; 7: 254–67.

29 Kawanishi H, Matsui Y, Ito M et al. Secreted CXCL1 is a potential mediator and marker of the tumor invasion of bladder cancer. Clin Cancer Res 2008; 14: 2579–87.

30 Makito M, Adrienne L, Steve G et al. Chemokine (C-X-C) ligand1 (CXCL1) protein expression is increased in aggressive bladder cancer. BMC Cancer 2013; 13: 322.

31 Gui SL, Teng LC, Wang SQ et al. Overexpression of CXCL3 can enhance the oncogenic potential of prostate cancer. Int Urol Nephrol 2016; 14: 701–9.

32 See AL, Chong PK, Lu SY et al. CXCL3 is a potential target for breast cancer metastasis. Curr Cancer Drug Targets 2014; 14: 294–309.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The strategy for analysis of genes regulated by miR-139-5p and miR-139-3p.

Fig. S2. Strategy for investigating the downstream miR-139-MMP11 axis in bladder cancer.

Fig. S3. Molecular mechanisms of miR-139-5p and miR-139-3p silencing in BC cells.

Fig. S4. Images of wound healing assay and matrigel invasion assay.

Fig. S5. Effects of co-transfection of miR-139-5p and miR-139-3p on cell proliferation, migration and invasion assays.

Fig. S6. Expression of miR-139-5p/miR-139-3p/MMP11 downstream genes in BC cells.

Table S1. Patients’ characteristics.

Table S2. Patient characteristics of tissue microarray patients.

Table S3. Cox proportional analysis for the prediction of BC overall survival.

Table S4. Relationships between MMP11 expression and clinicopathological factors in tissue microarray.

Table S5. Total 116 genes of significantly enriched annotations (downregulated genes in miR-139 and si-MMP11 transfectants).

Table S6. Primer Sequences for PCR experiments of the genes.