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# 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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**Our recent study of the microRNA (miRNA) expression signature of bladder cancer (BC) by deep-sequencing revealed that two miRNA, *microRNA-139-5p*/*microRNA-139-3p* were significantly 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 ($P = 0.029$). Down-regulated *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.**

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] 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.[5,6] A substantial amount of evidence has demonstrated that aberrantly expressed miRNA can act as oncogenes or tumor suppressors. These miRNA can disrupt tightly controlled RNA networks in cancer cells.[7] 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.[5] 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 strands derived from the miRNA duplex actually have biological functions in normal or cancer cells. Our recent study of the miRNA expression signature of BC by deep-sequencing showed that *miR-139-5p* (guide strand) and *miR-139-3p* (passenger strand) derived from *pre-miR-139* were downregulated in BC tissues.

The aim of the present study was to investigate the functional significance of *miR-139-5p*/*miR-139-3p* and to identify the molecular targets that are regulated by these miRNA in BC cells. Our data demonstrated that restoration of *miR-139-5p*/*miR-139-3p* significantly inhibited cancer cell viability through targeting of the *matrix metalloprotease 11* (*MMP11*). We expect that this analysis will provide novel insights into the potential molecular mechanisms of miRNA-RNA networks

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°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*

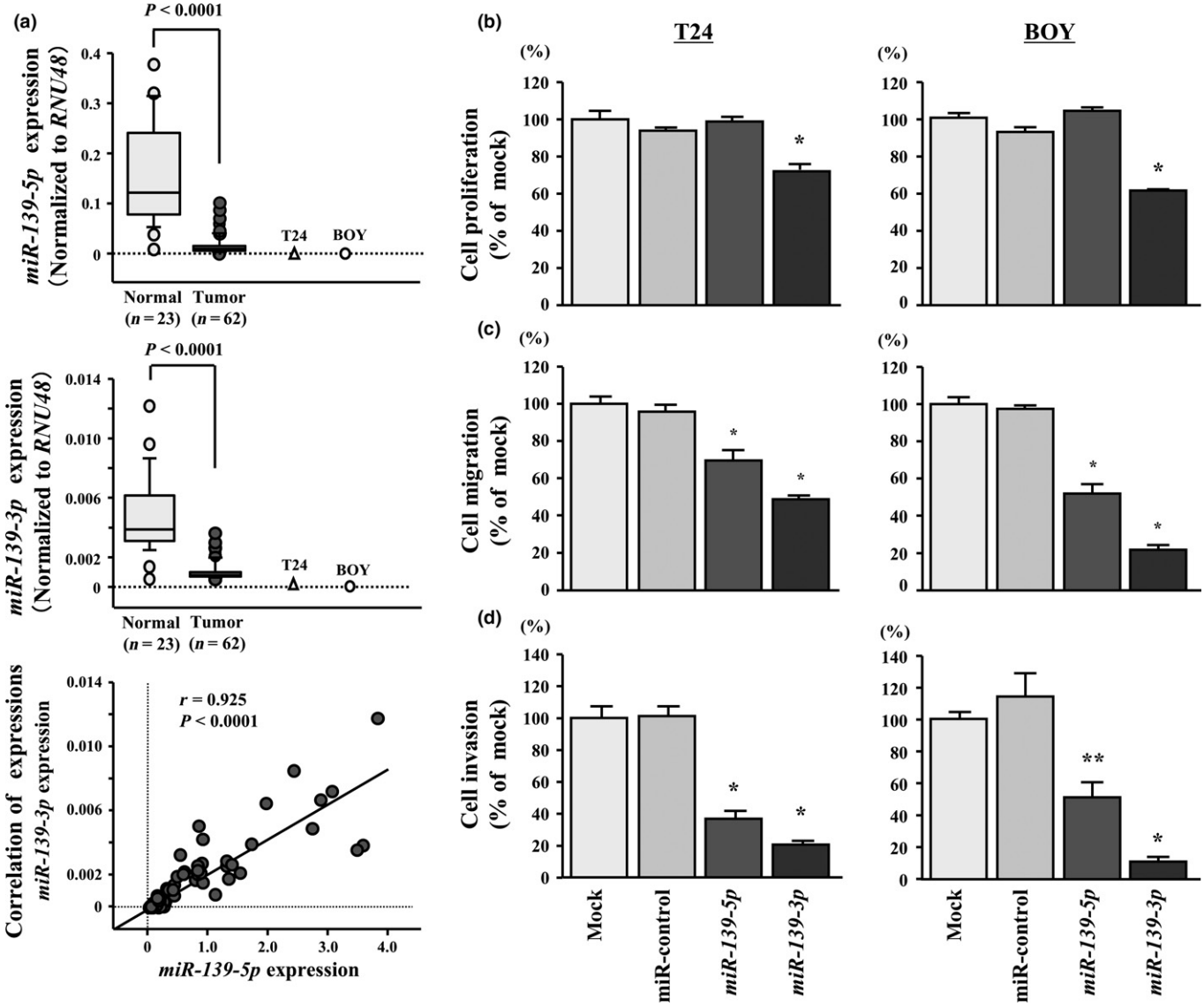


**Fig. 1.** The expression levels of *miR-139-5p* and *miR-139-3p* and their effects in BC cells. (a) Expression levels of *miR-139-5p* and *miR-139-3p* in BC tissues and BC cell lines and the correlated expression of *miR-139-5p* and *miR-139-3p* were determined by qRT-PCR. Data were normalized to *RNU48* expression. (b) Cell proliferation was determined by XTT assays 72 h after transfection with 10 nM *miR-139-5p* or *miR-139-3p* (*$P$ < 0.0001). (c) Cell migration activity was determined by wound-healing assays 48 h after transfection with10 nM *miR-139-5p* or *miR-139-3p* (*$P$ < 0.0001). (d) Cell invasion activity was determined by Matrigel invasion assays 48 h after transfection with 10 nM *miR-139-5p* or *miR-139-3p* (*$P$ < 0.0001; **$P$ < 0.005).

(product ID: Hs99999908_m1; Applied Biosystems) and *RNU48* (product ID: 001006; Applied Biosystems) as internal controls.

**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 PmeI restriction sites in the 3′-UTR of *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure

**Table 1. Identification of *miR-139-5p* and *miR-139-3p* commongenes**

| Entrez gene ID | Gene symbol | Description | GEO | | Expression in T24 transfectant (log 2 ratio) | |
|---|---|---|---|---|---|---|
| | | | Fold change | *P*-value | *miR-139-5p* | *miR-139-3p* |
| 4320 | *MMP11* | *Matrix metalloprotease 11* | 15.05 | 4.38E-05 | −0.77 | −0.95 |
| 26579 | *MYEOV* | *Myeloma overexpressed* | 7.18 | 1.00E-04 | −0.78 | −2.50 |
| 27283 | *TINAG* | *Tubulointerstitial nephritis antigen* | 5.85 | 6.05E-05 | −0.51 | −2.98 |
| 54626 | *HES2* | *Hes family bHLH transcription factor 2* | 5.47 | 8.23E-04 | −1.11 | −0.90 |
| 8091 | *HMGA2* | *High mobility group AT-hook 2* | 4.65 | 7.32E-05 | −0.90 | −2.31 |
| 157570 | *ESCO2* | *Establishment of sister chromatid cohesion N-acetyltransferase 2* | 4.08 | 4.98E-05 | −0.64 | −0.59 |
| 22979 | *EFR3B* | *EFR3 homolog B* | 3.49 | 1.64E-04 | −0.78 | −0.74 |
| 5209 | *PFKFB3* | *6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3* | 3.26 | 1.85E-04 | −0.57 | −0.69 |
| 84696 | *ABHD1* | *Abhydrolase domain containing 1* | 2.85 | 3.54E-04 | −0.66 | −1.40 |
| 114794 | *ELFN2* | *Extracellular leucine-rich repeat and fibronectin type III domain containing 2* | 2.69 | 5.61E-03 | −0.76 | −2.09 |
| 1051 | *CEBPB* | *CCAAT/enhancer binding protein (C/EBP), beta* | 2.42 | 4.46E-04 | −0.88 | −1.35 |
| 3718 | *JAK3* | *Janus kinase 3* | 2.32 | 3.54E-04 | −0.93 | −0.76 |
| 3690 | *ITGB3* | *Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)* | 1.85 | 1.26E-03 | −1.19 | −3.17 |
| 23150 | *FRMD4B* | *FERM domain containing 4B* | 1.84 | 4.87E-03 | −0.52 | −1.09 |
| 85377 | *MICALL1* | *MICAL-like 1* | 1.80 | 1.68E-02 | −1.12 | −1.54 |
| 55689 | *YEATS2* | *YEATS domain containing 2* | 1.69 | 9.42E-05 | −0.75 | −1.06 |
| 5829 | *PXN* | *Paxillin* | 1.69 | 3.49E-03 | −0.56 | −2.20 |
| 79858 | *NEK11* | *NIMA-related kinase 11* | 1.53 | 2.81E-04 | −0.60 | −0.70 |
| 10616 | *RBCK1* | *RanBP-type and C3HC4-type zinc finger containing 1* | 1.51 | 4.87E-03 | −0.73 | −1.10 |
| 9526 | *MPDU1* | *Mannose-P-dolichol utilization defect 1* | 1.44 | 1.54E-02 | −0.75 | −0.61 |
| 2979 | *GUCA1B* | *Guanylate cyclase activator 1B (retina)* | 1.43 | 2.23E-02 | −0.65 | −0.63 |
| 55275 | *VPS53* | *Vacuolar protein sorting 53 homolog* | 1.30 | 2.35E-03 | −0.79 | −0.54 |

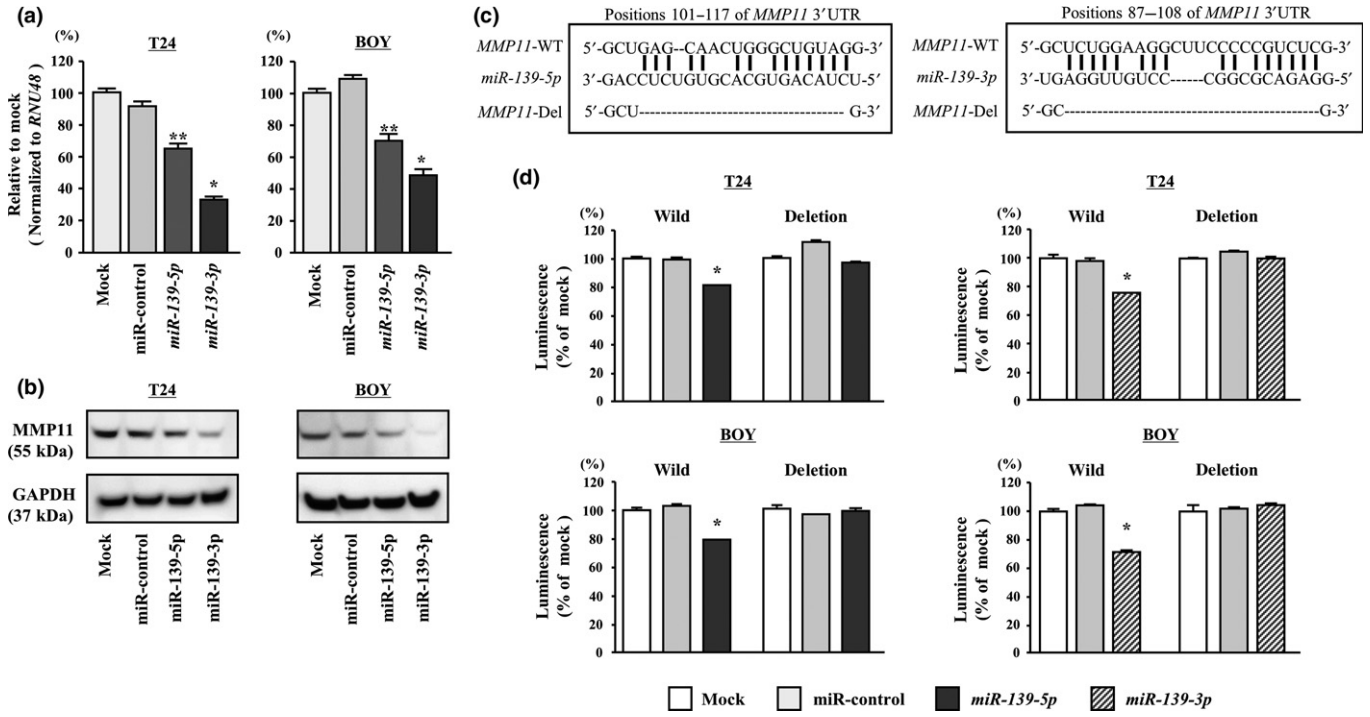


**Fig. 2.** Direct regulation of *MMP11* by *miR-139-5p* or *miR-139-3p* (a) *MMP11* mRNA expression was evaluated by qRT-PCR 72 h after transfection with 30 nM *miR-139-5p* and *miR-139-3p*. *RNU48* was used as an internal control (*$P < 0.0001$; **$P < 0.001$). (b) MMP11 protein expression was evaluated by Western blotting 72 h after transfection with 30 nM *miR-139-5p* and *miR139-3p*. GAPDH was used as a loading control. (c) *miR-139-5p* or *miR-139-3p* binding site in the 3′-UTR of *MMP11* mRNA. (d) Luciferase reporter assays using vectors encoding a putative *miR-139-5p* and *miR-139-3p* target site of the *MMP11* 3′-UTR. *Renilla* luciferase values were normalized to firefly luciferase values (*$P < 0.001$).

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 MMP11 (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. Matsushita), 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 *MMP11* 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 *PED2A* 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

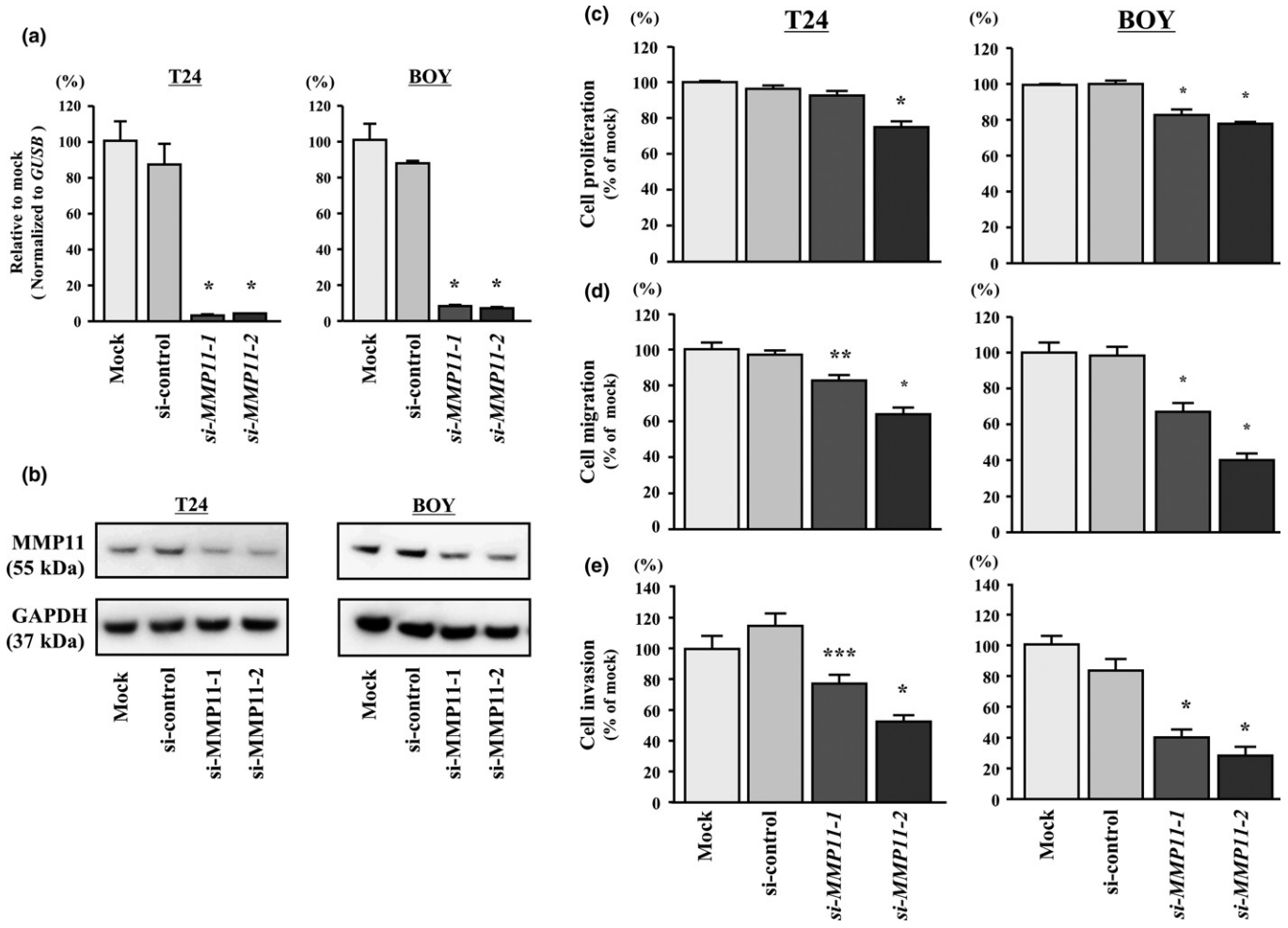


**Fig. 3.** *MMP11* mRNA and protein expression after *si-MMP11* transfection and effects of silencing *MMP11* in BC cell lines. (a) *MMP11* mRNA expression levels were evaluated by qRT-PCR 72 h after transfection with 10 nM *si-MMP11*. *GUSB* was used as an internal control (*$P < 0.0001$). (b) MMP11 protein expression was evaluated by Western blotting 72 h after transfection with *si-MMP11* (10 nM). GAPDH was used as a loading control. (c) Cell proliferation was determined by XTT assays (*$P < 0.0001$). (d) Cell migration activity was determined by wound healing assays (*$P < 0.0001$; **$P < 0.001$). (e) Cell invasion activity was determined by Matrigel invasion assays (*$P < 0.0001$; ***$P < 0.05$).

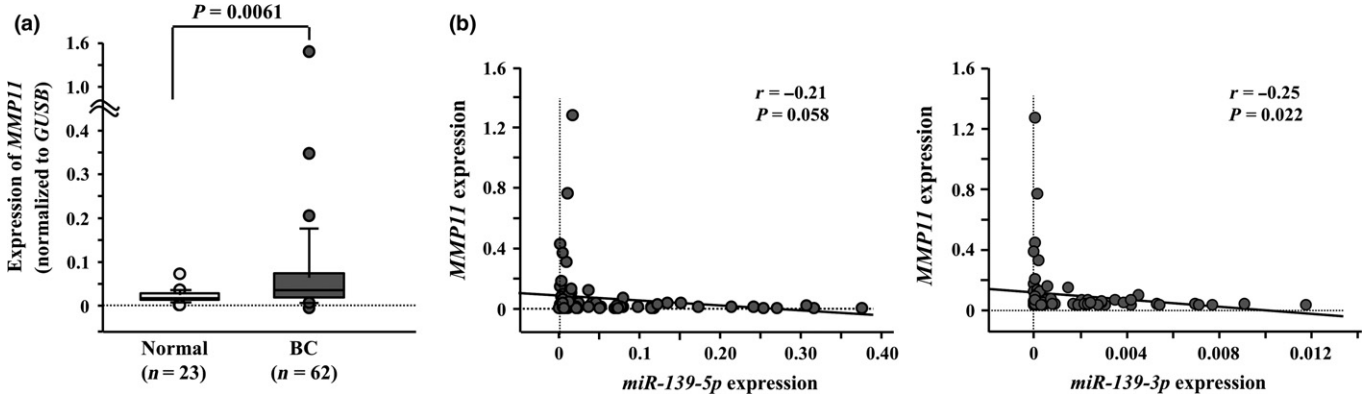


**Fig. 4.** The expression level of *MMP11* mRNA in BC clinical specimens. (a) *MMP11* mRNA expression in 62 BC specimens and 23 non-BC specimens. *GUSB* was used as an internal control. (b) Inverse correlations between *MMP11* mRNA and *miR-139-5p* and *miR-139-3p* expression.

*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.[13] 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). XTT, 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*

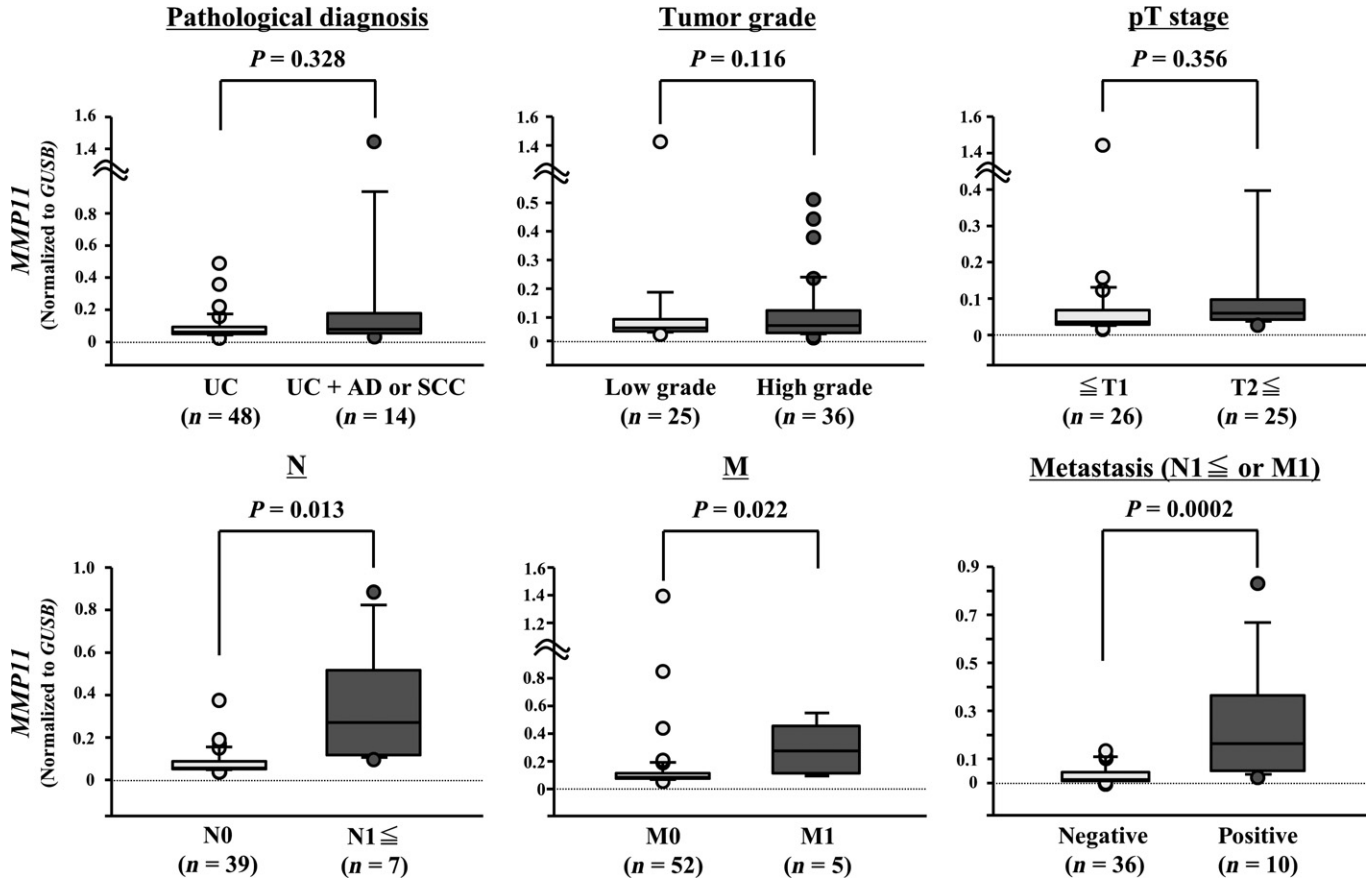


**Fig. 5.** The association between the expression level of *MMP11* with clinicopathological parameters. The association of *MMP11* expression with clinicopathological parameters was determined with the Mann–Whitney *U*-test. *GUSB* was used as an internal control.

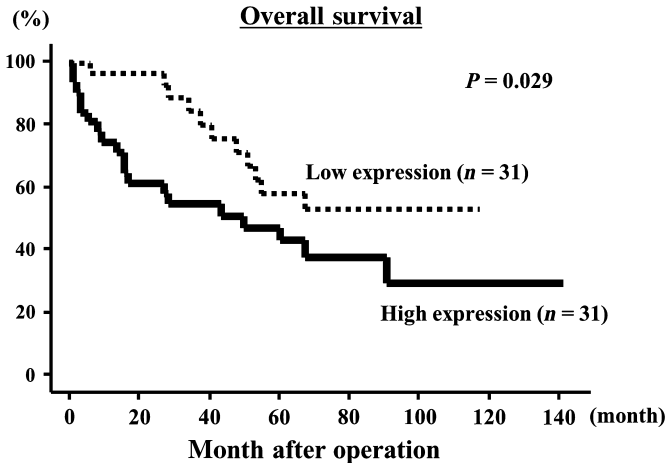


**Fig. 6.** The association between the expression level of *MMP11* and overall survival. Kaplan–Meier survival curves for overall survival rate based on *MMP11* expression in 62 BC patients. *P*-values were calculated using the log-rank test.

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

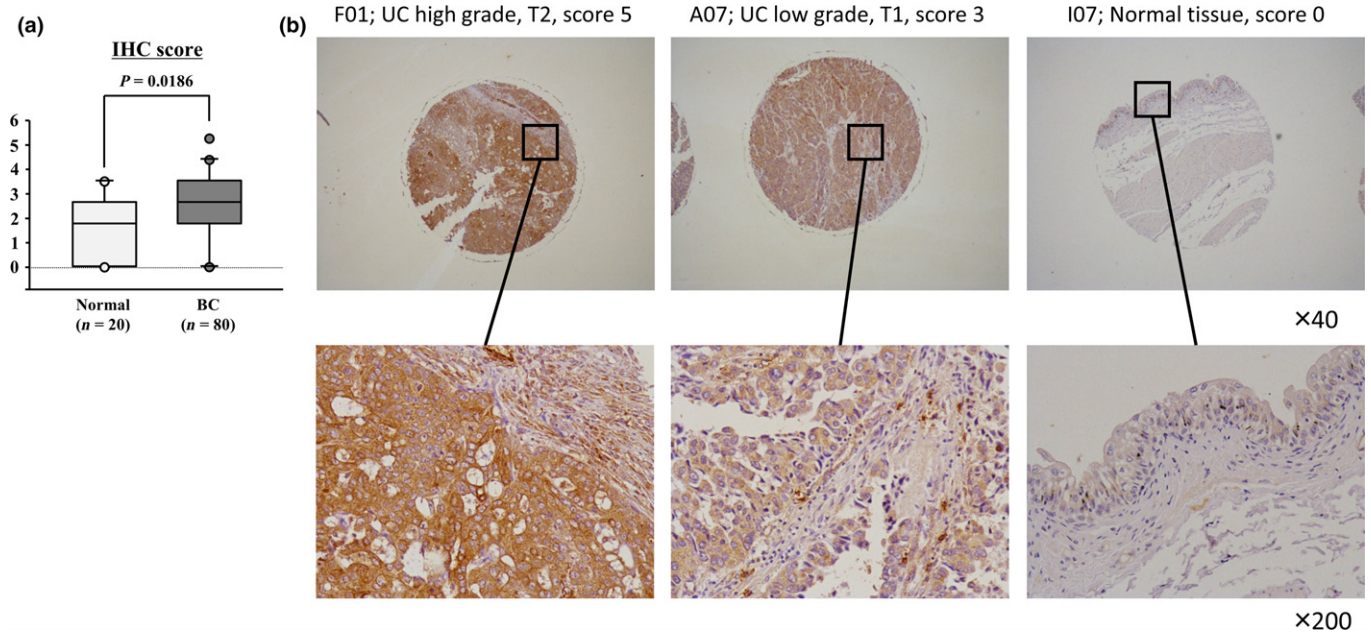


**Fig. 7.** Immunohistochemical staining of MMP11 in tissue specimens. (a) There was a significant difference in the expression score of MMP11 of 80 BCs in comparison with 20 NBEs. (b) Immunohistochemical staining of MMP11 in tissue specimens (magnification ×40 and ×200). Immunoreactivity for MMP11 was 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)**

| Entrez Gene ID | Gene symbol | Description | GEO | | Expression in transfectant ($log_2$ ratio) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Fold change | *P*-value | T24 *si-MMP11-2* | BOY *si-MMP11-2* | T24 *miR-139-5p* | T24 *miR-139-3p* |
| 147841 | *SPC24* | *SPC24, NDC80 kinetochore complex component* | 15.185 | 4.98E-05 | −1.356 | −1.650 | −0.092 | −0.674 |
| 4320 | *MMP11* | *Matrix metallopeptidase 11 (stromelysin 3)* | 15.051 | 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* | *Denticleless 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 | *KLC3* | *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 | *POC1A* | *POC1 centriolar protein A* | 5.113 | 4.38E-05 | −0.530 | −0.558 | −0.139 | −1.259 |
| 1869 | *E2F1* | *E2F transcription factor 1* | 5.036 | 4.67E-05 | −0.730 | −1.332 | −0.118 | −1.049 |

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.[8,10–12,14]

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 miRNA 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-tumour 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. S5). 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 *CCDN1*, *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 deferent 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).[21] Unlike other MMP, *MMP11* does not cleave any classical ECM components, suggesting that it plays indirect roles in ECM remodeling in cancer cells.[22] Previous studies showed that high expression of *MMP11* correlated with poor prognosis and predicted recurrence in breast cancer.[23] A recent study of colorectal cancer showed that *MMP11* expression was a predictive survival marker of colorectal cancer patients.[24] Moreover, oncogenic roles of *MMP11* were reported in several cancers, including cervical cancer and gastric cancer.[25,26] 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.[27]

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.[28] *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.[28] Past studies showed that *CXCL1* modulates the invasive abilities of BC cells and this chemokine may be a potential biomarker for invasive bladder cancer.[29] Expression of CXCL1 is upregulated in more aggressive phenotype of BC and high expression of CXCL1 has been associated with reduced disease-free survival.[30] 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.[31] Moreover, *CXCL3* is involved in the metastasis of breast cancer.[32] 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.

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## Disclosure Statement

The authors have no conflicts of interest to declare.

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## 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.