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Regulation of *ITGA3* by the dual-stranded *microRNA-199* family as a potential prognostic marker in bladder cancer

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Background: Based on the microRNA (miRNA) signature of bladder cancer (BC) by deep sequencing, we recently found that several double-stranded mature miRNAs derived from the same pre-miRNAs were sufficiently expressed and acted as tumour suppressors by regulating common target genes in BC. Our deep-sequencing signature of BC showed that all *miR-199* family members (*miR-199a-3p/-5p* and *miR-199b-3p/-5p*) were also downregulated. We hypothesised that these miRNAs may function as tumour suppressors by regulating common target genes.

Methods: Functional assays of BC cells were performed using transfection of mature miRNA. *In silico* analyses and luciferase reporter analyses were applied to identify target genes of these miRNAs. The overall survival of patients with BC in The Cancer Genome Atlas (TCGA) database was evaluated by the Kaplan–Meier method.

Results: Restoration of these miRNAs significantly inhibited cell migration and invasion in BC cells. Integrin α3 (*ITGA3*) was directly regulated by these miRNAs. The Cancer Genome Atlas database showed that patients with low *pre-miR-199* family (*miR-199a-1/-2* and *miR-199b*) expression exhibited significantly poorer overall survival compared with patients with high *pre-miR-199* family expression.

Conclusions: *miR-199* family miRNAs functioned as tumour suppressors in BC cells by targeting *ITGA3* and might be good prognostic markers for predicting survival in patients with BC.

Bladder cancer (BC) is the ninth most commonly diagnosed cancer worldwide in 2012 (Torre *et al*, 2015; Antoni *et al*, 2017). In the United States of America, it was estimated that ~76 960 cases were diagnosed and 16 390 patients died in 2016 (Siegel *et al*, 2016). Bladder cancer can be classified into two groups: non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). More than 70% of patients with BC are diagnosed with NMIBC (Miller *et al*, 2016). However, patients with NMIBC tend to have a high rate of recurrence, as high as 50–70% (Kaufman *et al*, 2009). Patients with localised MIBC are managed by radical cystectomy. However, despite potential curative surgery, ~50% of patients develop metastatic disease within only 2 years (Sternberg *et al*, 2013). Patients with advanced BC are generally treated with systemic combination chemotherapy of cisplatin and gemcitabine; however, the therapeutic effects are insufficient (Kaufman *et al*, 2009; Sternberg *et al*, 2013). Most clinical trials of chemotherapeutics with other drugs for advanced BC have shown limited benefits and a short median survival period of only ~9.3–15.8 months (Bellmunt *et al*, 2012; De Santis *et al*, 2012). Moreover, there are no more reliable markers other than urine cytology for BC diagnosis, and this approach has relatively low sensitivity (30–40%) (Enokida *et al*, 2016). Therefore, novel prognostic markers and effective treatment strategies based on RNA network studies are urgently needed to improve outcomes in patients with BC.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs of 19–22 nucleotides in length that negatively regulate protein-

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coding genes by binding to the 3′-untranslated region (UTR) of the target mRNA, thereby inhibiting transcriptional or post-transcriptional expression (Bartel, 2004; Carthew and Sontheimer, 2009). Previous studies have demonstrated that miRNAs are aberrantly expressed in various types of cancer and have the critical roles in cancer cell behaviour (Di Leva and Croce, 2010). Therefore, the detection of aberrantly expressed miRNAs and their target genes could provide important insights into the elucidation of miRNA-mediated oncogenic pathways.

In miRNA biogenesis, the guide-strand of miRNA integrates into the RNA-induced silencing complex (RISC), whereas the passenger strand is inactivated through degradation (Chendrimada *et al*, 2007; Carthew and Sontheimer, 2009). However, our previous analysis of miRNA signatures in BC by deep sequencing revealed that the opposite strands of *miR-144-5p/-3p*, *miR-145-5p/-3p*, and *miR-139-5p/-3p* function as dual-strand tumour-suppressor miRNAs (Matsushita *et al*, 2015, 2016;Yonemori *et al*, 2016). Based on our additional analysis of the miRNA signature, we also found another set of downregulated miRNAs, the *miR-199* family (*miR-199a-3p/-5p* derived from *pre-miR-199a-1/-2* and *miR-199b-3p/-5p* derived from *pre-miR-199b*) (Itesako *et al*, 2014). The sequence of *miR-199a-3p* (guide strand) is the same as that of *miR-199b-3p* (guide strand), and the sequence of *miR-199a-5p* (passenger strand) is very similar to that of *miR-199b-5p* (passenger strand) (only two bases differ). Therefore, we hypothesised that these miRNAs may function as tumour suppressors by regulating common target genes. Several previous studies have shown that *miR-199* family members function as tumour suppressors in various types of cancer (Hu *et al*, 2014; Li *et al*, 2015; Sun *et al*, 2016). With regard to *miR-199a-5p*, some reports have shown that this miRNA functions as a tumour suppressor in BC (Su *et al*, 2013; Song *et al*, 2015). However, the role of *miR-199* family members in BC is still unclear.

The aim of this study was to investigate the antitumour effects of *miR-199* family members and to determine their regulatory targets in BC. The elucidation of molecular targets regulated by tumour-suppressive *miR-199* family member will provide important new insights into the potential mechanisms of BC oncogenesis and progression.

MATERIALS AND METHODS

Clinical specimens and cell culture. The tissue specimens for quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) were collected from BC patients ($n = 32$) at Kagoshima University Hospital between 2004 and 2013. Normal bladder epithelia (NBE) ($n = 12$) were derived from patients with noncancerous disease. The specimens were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer tumour node metastasis classification and histologically graded (Sobin and Compton, 2010). Our study was approved by the Bioethics Committee of Kagoshima University; written prior written consent and approval were obtained from all patients. Patient details and clinicopathological characteristics are summarised in Supplementary Table 1.

We used human BC cell lines: T24 and BOY. These cell lines were described in our previous studies (Itesako *et al*, 2014).

Tissue collection and RNA extraction. Tissues were immersed in RNA later (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until RNA extraction. Total RNA, including miRNA, was extracted using a mirVana miRNA Isolation Kit (Thermo Fisher Scientific) following the manufacturer's protocol.

Quantitative real-time reverse transcription–polymerase chain reaction. Stem-loop RT–PCR (TaqMan MicroRNA Assays; P/N: 000498 for *miR-199a-5p*, P/N: 000500 for *miR-199b-5p*, and P/N: 002304 for *miR-199a-3p*/*miR-199b-3p*; Applied Biosystems, Foster City, CA, USA) was used to quantify miRNAs according to previously published conditions (Ichimi *et al*, 2009). We used human *RNU48* (P/N: 001006; Applied Biosystems) as an internal control, and the ΔCt method was used to calculate the fold changes. As for integrin α3 (*ITGA3*), we applied a SYBR-green quantitative PCR-based array approach; each sequence is listed in Supplementary Table 2. The experimental procedures followed the protocol recommended by the manufacturer. Quantitative real-time reverse transcription–polymerase chain reaction was performed with 500 ng of total RNA using Power SYBR Green Master Mix (cat. no. 4367659; Applied Biosystems) on a 7300 Real-time PCR System (Applied Biosystems). The specificity of amplification was monitored using the dissociation curve of the amplified product. All data values were normalised to *GUSB*, and the ΔCt method was used to calculate the fold change.

Transfection with mature miRNA and siRNA. As described elsewhere (Ichimi *et al*, 2009), T24 and BOY cells were transfected with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) and Opti-MEM (Thermo Fisher Scientific) with 10 nM mature miRNA or small interfering RNA (siRNA). Mature miRNA precursors (*hsa-miR-199a-5p*, product ID PM10893; *hsa-miR-199b-5p*, product ID: PM10553; *hsa-miR-199a/b-3p*, product ID: PM11779; Thermo Fisher Scientific) and negative-control miRNA (product IDs: AM17110, AM17111; Thermo Fisher Scientific) were used in gain-of-function experiments, whereas *ITGA3* siRNA (cat nos. HSS105529 and HSS105531; Thermo Fisher Scientific) and negative-control siRNA (D-001810-10; Thermo Fisher Scientific) were used in loss-of-function experiments.

Cell proliferation, migration, and invasion assays. Cell proliferation was determined by XTT assay according to the manufacturer's instructions. Cell migration activity was evaluated with wound-healing assays. Cell invasion assays were performed using modified Boyden chambers consisting of Transwell-precoated Matrigel membrane filter inserts with 8-mm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). The experimental procedures were performed as described in our previous studies (Tatarano *et al*, 2011; Yoshino *et al*, 2011). All experiments were performed in triplicate.

Western blot analysis. After transfection (72 h), protein lysates were separated on NuPAGE on 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes. Immunoblotting was carried out with diluted (1 : 1000) anti-ITGA3 antibodies (HPA008572; Sigma-Aldrich, St Louis, MO, USA) and anti-β-actin antibodies (bs-0061R; Bioss, Woburn, MA, USA). Specific complexes were visualised using an echochemiluminescence detection system (GE Healthcare, Little Chalfont, UK) as described previously (Yoshino *et al*, 2011).

Immunohistochemical analysis. A tissue microarray of BC samples was obtained from US Biomax Inc. (product ID: BL 1002; Rockville, MD, USA). Detailed information on all tumour specimens can be found at http://www.biomax.us/index.php. The tissue microarray was immunostained following the manufacturer's protocol with an Ultra Vision Detection System (Thermo Scientific). Primary rabbit polyclonal antibodies against ITGA3 (HPA008572; Sigma-Aldrich) were diluted 1 : 20. Immunostaining was evaluated according to the scoring method described previously (Yoshino *et al*, 2011). Each case was scored on the basis of the intensity and area of staining. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic field; 1+, <30% of cells stained positive; 2+, 30–60% stained positive; 3+, >60% stained positive. The

immunostaining scores (intensity + extent) were combined and analysed. All samples were independently scored by two of the authors (T Sakaguchi and K Miyamoto), who were blinded to the patient status.

***In silico* analysis for the identification of genes regulated by *miR-199a-3p/-5p* and *miR-199b-3p/-5p*.** To obtain candidate target genes regulated by *miR-199a-3p/-5p* and *miR-199b-3p/-5p*, we used TargetScan database Release 7.0 (http://www.targetscan.org). These candidate target genes data were adapted to the KEGG pathway categories by the GENECODIS program (http://genecodis.cnb.csic.es). Additionally, the Gene Expression Omnibus (GEO) database (accession numbers: GSE11783 and GSE31684) was used to identify upregulated genes in BC specimens.

Plasmid construction and dual-luciferase reporter assays. Partial wild-type sequences of the 3′-UTR of *ITGA3* or sequences with deletion of the *miR-199a-5p*/*miR-199b-5p* target sites and *miR-199a-3p*/*miR-199b-3p* target site were inserted between the *Xho*I and *Pme*I restriction sites in the 3′-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). T24 and BOY cells were transfected with 50 ng vector and 10 nM *miR-199a-5p* or *miR-199b-5p* or *miR-199a/b-3p*. The procedure for dual-luciferase reporter assay was described previously (Yoshino *et al*, 2013a).

Analysis of the BC cohort in TCGA data sets. The Cancer Genome Atlas (TCGA) cohort database was used to analyse *ITGA3* mRNA expression levels in normal and BC samples and to determine if there was a clinical relationship between *miR-199a/b* or *ITGA3* expression and overall survival. RNA-seq expression data (normalised RSEM) and miRNA-seq data (reads per million mapped reads) were used for gene and miRNA quantification (Li and Dewey, 2011). mRNA expression by whole-exome sequencing data was available for 408 patients with BC. Full sequencing information and clinical information were acquired using cBioPortal (http://www.cbioportal.org/public-portal/) and TCGA (https://tcga-data.nci.nih.gov/tcga/) (Cerami *et al*, 2012; Gao *et al*, 2013; Cancer Genome Atlas Research N, 2014). This study meets the publication guidelines provided by TCGA (http://cancergenome.nih.gov/publications/publicationguidelines).

Statistical analysis. The relationships between two groups were analysed using Mann–Whitney *U*-tests. The relationships between three variables and numerical values were analysed using Bonferroni-adjusted Mann–Whitney *U*-tests. Spearman's rank tests were used to evaluate the correlations between the expression of *miR-199a-5p* or *miR-199b-5p* and *miR-199a/b-3p*. χ^2 tests were used to analyse the relationships between *ITGA3* expression in Immunohistochemical (IHC) staining and clinicopathological characteristics. The overall survival of patients with BC from the TCGA cohort was evaluated by the Kaplan–Meier method. Patients were divided into two or three groups based on the number of patients in the cohort, and differences between the two groups were evaluated by log-rank tests. All analyses were carried out using Expert StatView software, version 5.0 (Cary, NC, USA).

RESULTS

Expression levels of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* in BC. First, we evaluated the expression levels of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* in BC tissues ($n = 32$) and NBE ($n = 12$) by qRT–PCR. The expression levels of these mature miRNAs were significantly reduced in BC tissues compared with those in the NBE ($P = 0.0032$, $P < 0.0001$, and $P < 0.0001$, respectively; Figure 1A). Spearman's rank test revealed significant positive correlations between the expression levels of these miRNAs (each $P < 0.0001$; Figure 1B). We found no significant relationships between the clinicopathological parameters (i.e.,

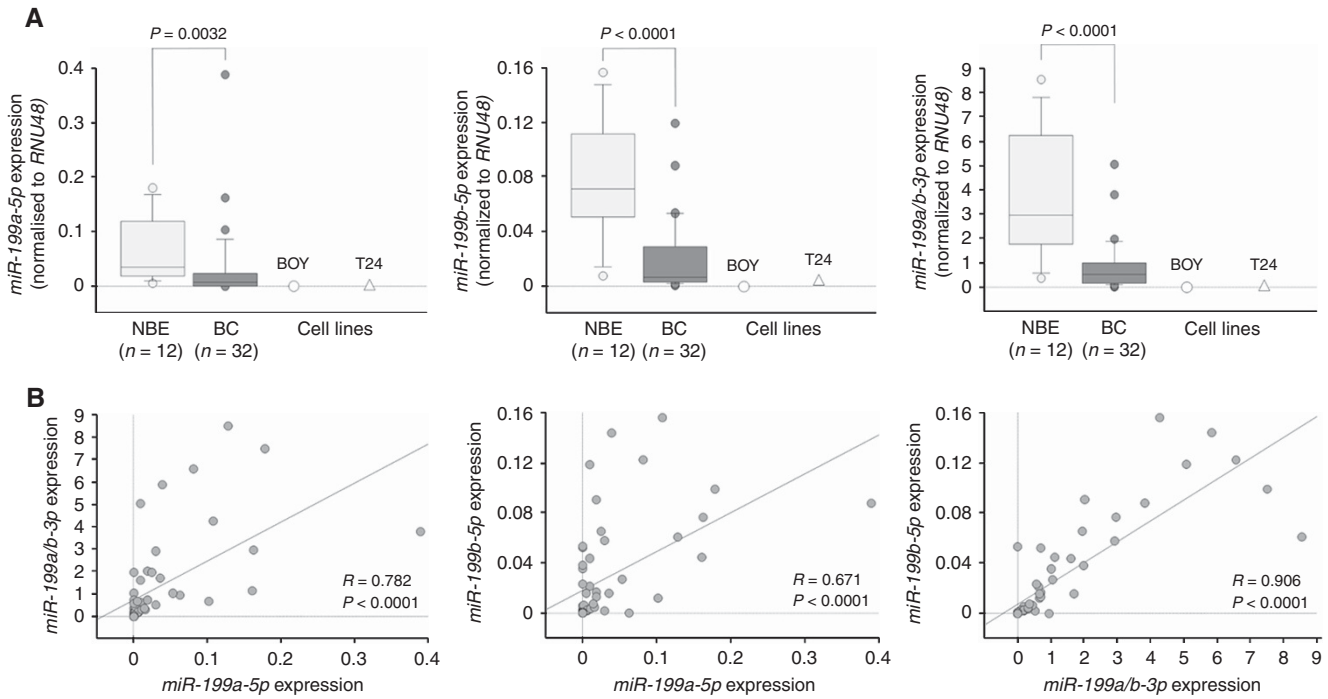

Figure 1. **The expression levels of miR-199 family members in clinical BC specimens.** (**A**) The expression levels of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p*, as determined by qRT–PCR, were significantly lower in BC specimens than in NBE specimens ($P = 0.0032$, $P < 0.0001$, and $P < 0.0001$, respectively). (**B**) Spearman's rank test demonstrated that these miRNAs expression levels were positively correlated (each $P < 0.0001$).

tumour stage, grade) and the expression levels of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* (data not shown).

Effects of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* restoration on cell proliferation, migration, and invasion activities in BC cell lines. We performed gain-of-function studies using T24 and BOY cells transfected with *miR-199a-5p*, *miR-199b-5p*, or *miR-199a/b-3p* to investigate their functional roles. XTT assays revealed significant inhibition of cell proliferation in T24 and BOY cells transfected with *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* in comparison with mock or miR-control transfectants (T24: $P<0.05$, $P=0.0007$, and $P<0.0001$, respectively; BOY: $P<0.0001$, $P<0.0001$, and $P<0.05$, respectively; Figure 2A). Moreover, wound-healing assays demonstrated that cell migration activity was significantly inhibited in these miRNA transfectants in comparison with that in mock or miR-control transfectants (each $P<0.0001$; Figure 2B). Finally, Matrigel invasion assays demonstrated that the number of invading cells was significantly decreased in these miRNA transfectants compared with mock or miR-control transfectants (each $P<0.0001$; Figure 2C). These data suggested that *miR-199* family members functioned as tumour suppressors in BC cells.

Identification of common target genes regulated by *miR-199* family members in BC cells. Next, *in silico* analysis was used to gain additional insights into the molecular mechanisms and pathways regulated by tumour-suppressive *miR-199* family members in BC cells. Supplementary Figure 1 shows our strategy to narrow down the common target genes of *miR-199* family members. Candidate genes of *miR-199-5p* and *miR-199-3p* (4195 and 3013 genes, respectively) were identified using TargetScan database Release 7.0 (http://www.targetscan.org). Among these candidate genes, we identified 1127 common candidate target genes of *miR-199-5p* and *miR-199-3p*. Moreover, these candidate target genes were categorised into KEGG pathways using GENECODIS analysis (http://genecodis.cnb.csic.es). Our analysis indicated that these genes were implicated in 64 significantly enriched pathways (Supplementary Table 3). Among these pathways, we focused on 'pathways in cancer', because the greatest number of genes (29 genes) was listed within this pathway. Of these 29 genes, 10 genes were upregulated in the GEO database (accession numbers: GSE11783 and GSE31684; Supplementary Table 4). Finally, among these 10 genes, we focused on the *ITGA3* gene because *miR-199* family members significantly inhibited cancer cell migration and invasion. Molecular target searches suggested that *ITGA3* was a promising candidate target regulated by the *miR-199* family in BC cells.

***ITGA3* was directly regulated by *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* in BC cells.** We performed qRT–PCR and western blot analyses to confirm that restoration of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* downregulated *ITGA3* mRNA expression in T24 and BOY cells. *ITGA3* mRNA levels were markedly and significantly reduced in these miRNA transfectants in comparison with those in mock or miR-control transfectants ($P<0.0001$ and $P=0.0002$; Figure 3A). Moreover, the expression

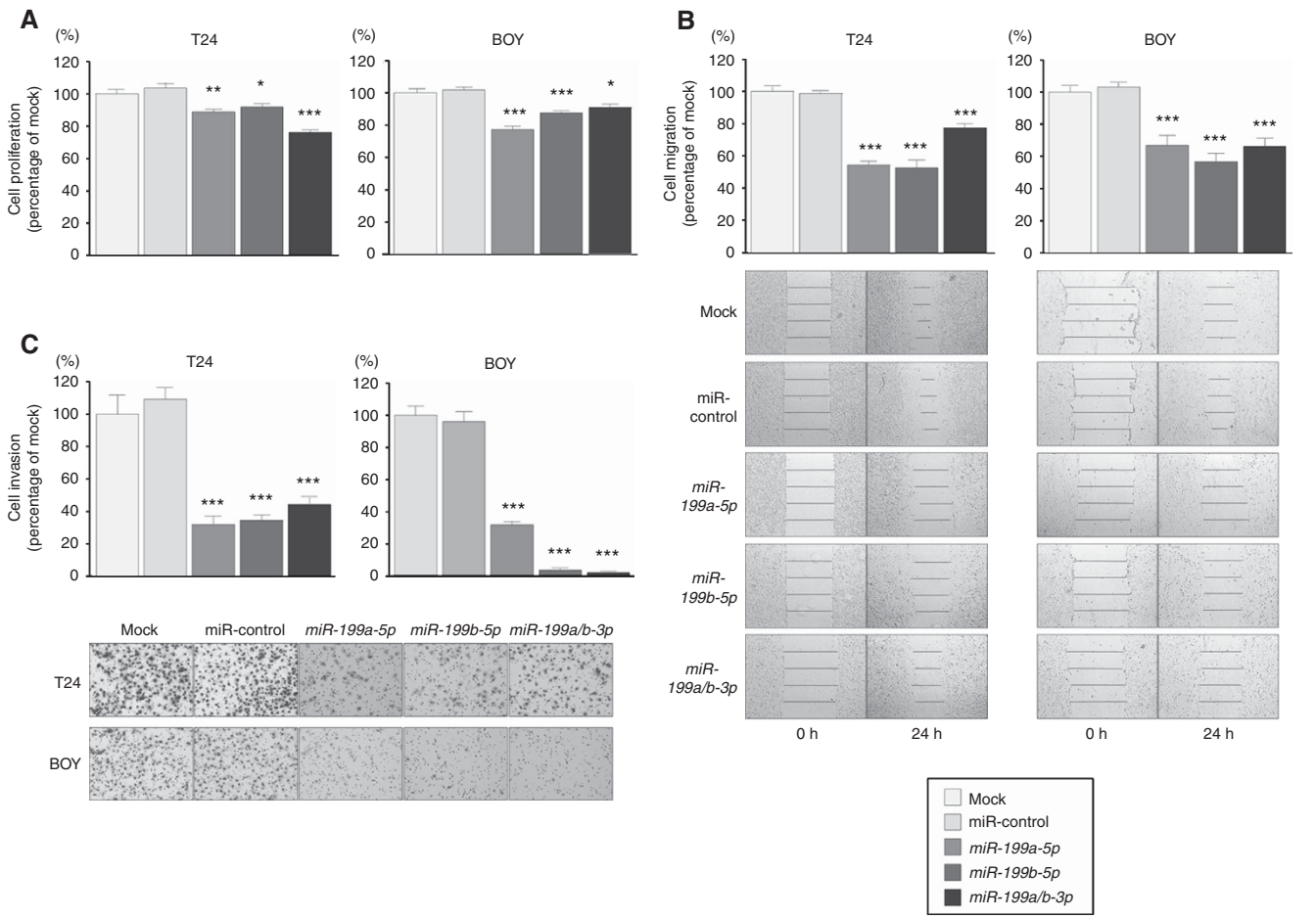

Figure 2. Effects of *miR-199a-5p*, *miR-199b-5p*, and *miR-199a/b-3p* transfection in BC cell lines. (**A**) Cell proliferation was determined by XTT assays. (**B**) Cell migration activity was determined with wound-healing assays. (**C**) Cell invasion activity was determined using Matrigel invasion assays. *$P<0.05$; **$P=0.0007$; ***$P<0.0001$.

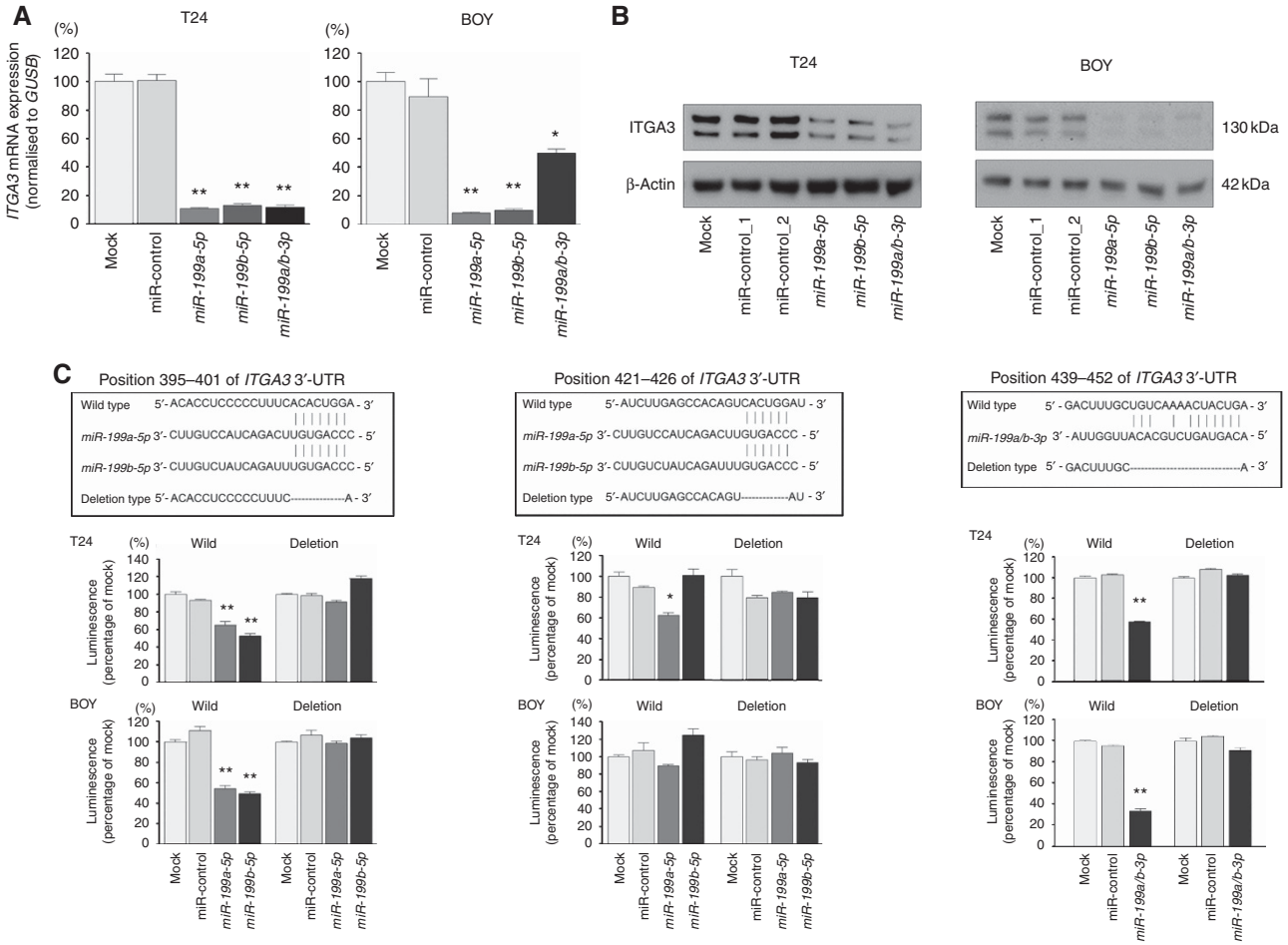

Figure 3. **Direct regulation of *ITGA3* by *miR-199a/b-5p* and *miR-199a/b-3p***. (**A**) The expression of *ITGA3* mRNA was significantly repressed in these miRNAs transfectants in comparison with that in mock or miR-control transfectants. *GUSB* was used as an internal control. $*P = 0.0002$; $**P < 0.0001$. (**B**) The expression of ITGA3 protein was markedly repressed in these miRNAs transfectants in comparison with that in mock or miR-control transfectants. β-Actin was used as a loading control. (**C**) Dual-luciferase reporter assays using vectors encoding putative miRNA target sites for wild-type or deleted regions. Normalised data were calculated as ratios of *Renilla*/firefly luciferase activities. The luminescence intensity was significantly reduced by co-transfection with *miR-199a/b-5p* or *miR-199a/b-3p* and the vector carrying the wild-type sequences at positions 395–401 and 439–452 in the *ITGA3* 3′-UTR. $*P < 0.0001$, $**P = 0.0001$.

of ITGA3 protein was markedly repressed in these miRNAs transfectants in comparison with mock or miR-control transfectants (Figure 3B). Two bands were observed on western blots and were found at the expected sizes for ITGA3 at around 130 kDa. These two bands could be the two isoforms of ITGA3 (α3A and α3B) (de de Melker *et al*, 1997). Another possibility is that aberrantly modified ITGA3 may be expressed. In fact, the expression of aberrantly glycosylated *ITGA3* in BC has been reported (Li *et al*, 2014, 2016a).

We then performed dual-luciferase reporter assays in BC cells to determine whether *ITGA3* was directly regulated by these miRNAs. The TargetScan database predicted that there were two binding sites for *miR-199a/b-5p* (positions 395–401 and 421–426) and one binding site for *miR-199a/b-3p* (position 439–452) in the *ITGA3* 3′-UTR. We used vectors encoding the partial wild-type sequence of the 3′-UTR of *ITGA3*, including the predicted *miR-199a/b-5p* and *miR-199a/b-3p* target sites. We found that the luminescence intensity was significantly reduced by co-transfection with these miRNAs and the vector carrying the wild-type sequences at positions 395–401 and 439–452 in the *ITGA3* 3′-UTR (each $P < 0.0001$), whereas transfection with the deletion vector (in which the binding site had been removed) and the wild-type sequence at positions 421–426 in the *ITGA3* 3′-UTR blocked the decrease in luminescence (Figure 3C). These data suggested that *miR-199a/b-5p* and *miR-199a/b-3p* were directly bound to specific sites in the 3′-UTR of *ITGA3* mRNA.

Effects of *ITGA3* knockdown on cell proliferation, migration, and invasion in BC cell lines. To investigate the functional role of *ITGA3* in the BC cells, we performed loss-of-function studies using cells transfected with two *si-ITGA3* constructs (*si-ITGA3_1* and *si-ITGA3_2*). We evaluated the knockdown efficiency of *si-ITGA3* transfection in BC cells. Quantitative real-time reverse transcription–polymerase chain reaction and western blot analyses showed that these siRNAs effectively downregulated *ITGA3* mRNA and protein expression in both cell lines (each $P < 0.0001$; Figures 4A and B). Additionally, in this experiment, we also observed two bands on western blot analysis for ITGA3, similar to the results described in the previous section. XTT assays demonstrated that BOY cell proliferation was inhibited in *si-ITGA3* transfectants in comparison with that in mock or si-control transfectants (each $P < 0.0001$). Although *si-ITGA3_1* inhibited cell proliferation significantly in T24 cells, no significant inhibition was observed in *si-ITGA3_2*-transfected T24 cells (Figure 4C). In contrast, wound-healing assays demonstrated that cell migration activity was significantly inhibited in *si-ITGA3* transfectants in comparison with that in mock or si-control

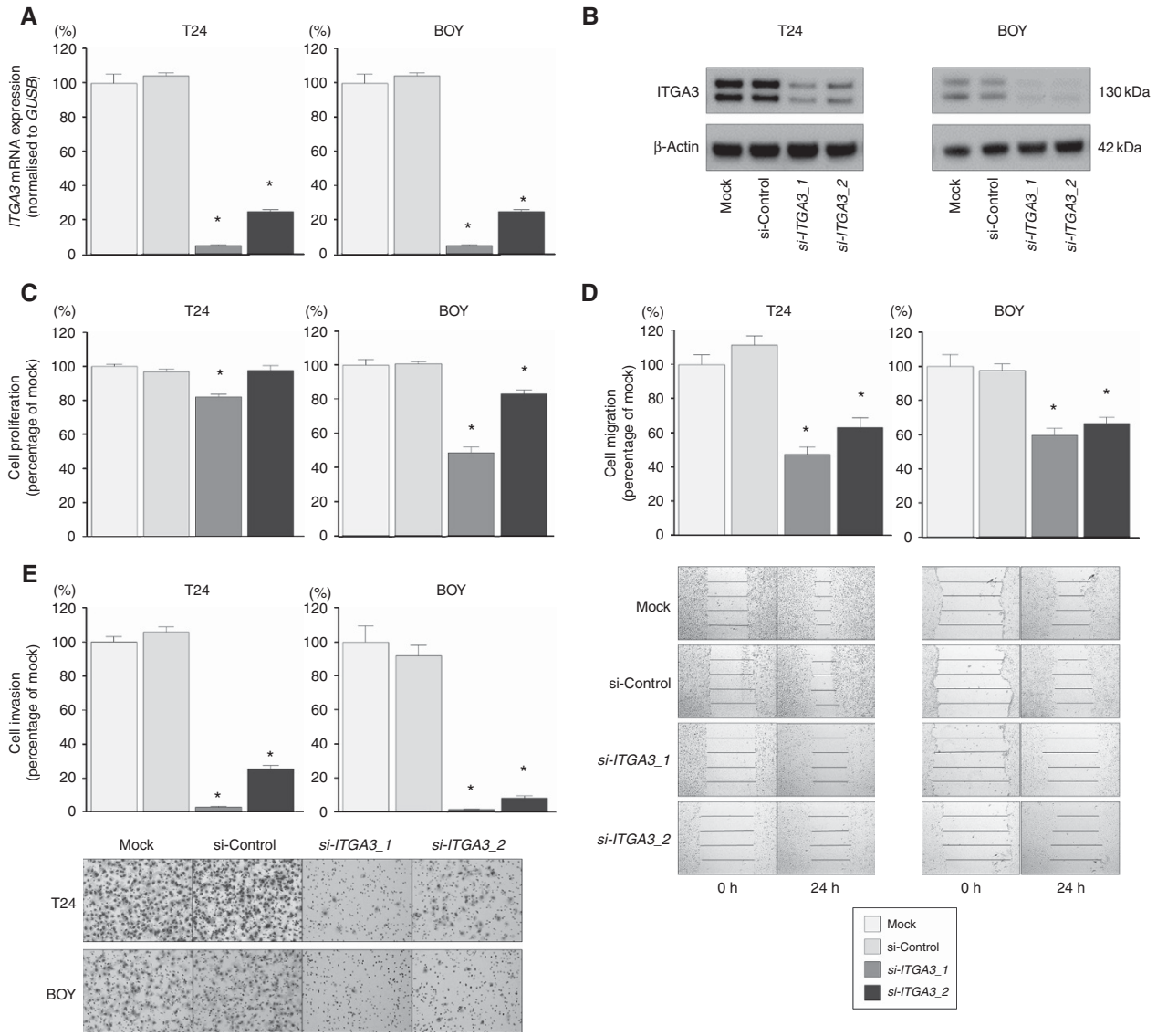

Figure 4. Effects of *si-ITGA3* transfection on BC cell lines. (**A**) The expression of *ITGA3* mRNA was significantly repressed in *si-ITGA3* transfectants in comparison with that in mock or si-control transfectants. *GUSB* was used as an internal control. *$P<0.0001$. (**B**) The expression of ITGA3 protein was markedly repressed in *si-ITGA3* transfectants in comparison with that in mock or si-control transfectants. β-Actin was used as a loading control. (**C**) Cell proliferation was determined by XTT assays. (**D**) Cell migration activity was determined using wound-healing assays. (**E**) Cell invasion activity was determined with Matrigel invasion assays. *$P<0.0001$.

transfectants (each $P<0.0001$; Figure 4D). Moreover, Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in *si-ITGA3* transfectants in comparison with that in mock or si-control transfectants (each $P<0.0001$; Figure 4E).

IHC analysis of ITGA3 in a tissue microarray. Next, we examined the expression level of ITGA3 in BC specimens by IHC staining. ITGA3 was strongly expressed in several tumour lesions, whereas low expression was observed in the normal tissue (Figures 5A–D). Tissue microarray analysis revealed that the IHC score of tumours was significantly higher than that of normal tissues (Mann–Whitney *U*-test; $P=0.0038$, χ^2 test; $P<0.0001$; Figure 5E, upper and Supplementary Table 5). In addition, we also found that ITGA3 expression in T2 or greater BCs was significantly higher than that in T1 BCs ($P=0.048$; Figure 5E, lower). However, we found no significant difference between the ITGA3 expression score and other pathological parameters (histological grade and pathological diagnosis) of BC patients (Supplementary Table 5).

Analysis of the clinical BC specimens in TCGA data sets. We investigated *ITGA3* mRNA expression levels and the correlations between *pre-miR-199* family members and *ITGA3* expression in BC samples using the TCGA database. Among the BC cohort in TCGA, we investigated patients for whom *ITGA3* expression data could be obtained. The expression level of *ITGA3* was significantly upregulated in patients with BC ($n=408$) compared with that in normal patients ($n=19$; $P=0.0063$; Figure 6A). Specifically, there was a significant negative correlation between *miR-199a-1* or *miR-199a-2* or *miR-199b* and *ITGA3* expression ($P=0.0099$, $R=-0.128$; $P=0.0189$, $R=-0.117$; and $P=0.033$, $R=-0.106$, respectively; Figure 6B).

We examined the correlation of *pre-miR-199* family expression levels with overall survival using the TCGA database. Among the BC cohort in TCGA, we investigated 404 patients for whom

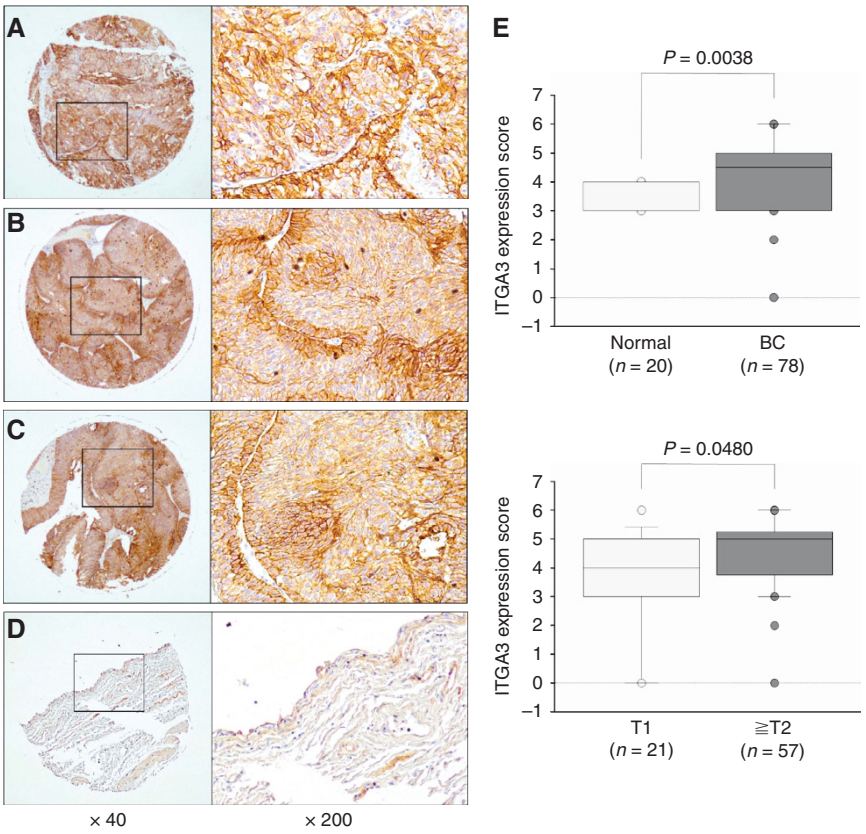

Figure 5. IHC staining of ITGA3 in tissue specimens. (**A**) Strong positive staining in a tumour lesion (grade 2, T3N0M0), (**B**) strong positive staining in a tumour lesion (grade 1, T2N0M0), (**C**) strong positive staining in a tumour lesion (grade 2, T1N0M0), and (**D**) weak positive staining in normal bladder tissue. (**E**) ITGA3 expression scores in IHC staining; upper, ITGA3 expression in normal bladder tissues and BC; lower, correlation between ITGA3 expression and tumour status in BC.

pre-miR-199 family expression and survival time data could be obtained. The cohort was divided into two groups based on the number of patients. Importantly, we found that patients with low *miR-199* family expression ($n = 202$) had poor survival compared with patients with high *miR-199* family expression ($n = 202$). Kaplan–Meier analysis showed that the low *miR-199a-1*, *miR-199a-2*, and *miR-199b* expression groups had significantly lower overall survival rates than patients with high expression of these pre-miRNA groups. The median overall survival times were 8.46, 8.46, and 8.48 months in the low *miR-199a-1*, *miR-199a-2*, and *miR-199b* expression groups *versus* 11.44, 11.44, and 11.41 months in the high expression groups ($P = 0.0028$, $P = 0.0020$, and $P = 0.0023$, respectively; Figure 6C). Next, we examined the correlation of *ITGA3* expression level with overall survival. Among the BC cohort in TCGA, we investigated 403 patients for whom *ITGA3* expression and survival time data could be obtained. The cohort was divided into three groups according to the number of patients. Kaplan–Meier analysis showed that patients in the high *ITGA3* expression group ($n = 134$) exhibited lower overall survival rates compared with those in the low expression group ($n = 134$); however, this difference was not significant ($P = 0.0823$). Moreover, there were no significant differences between the medium *ITGA3* expression group ($n = 135$) and the high or low expression groups (Supplementary Figure 2).

DISCUSSION

MicroRNAs are vitally important regulators that contribute to numerous physiological processes, such as cell proliferation, differentiation, development, and apoptosis (Croce, 2009; Di Leva and Croce, 2010). MicroRNAs are unique in their ability to regulate multiple protein-coding genes. Recently, bioinformatics predictions have indicated that miRNAs may regulate more than 60% of the protein-coding genes in the human genome (Friedman *et al*, 2009). Additionally, in human cancer, miRNAs are aberrantly expressed and have important roles in cancer initiation, development, and metastasis (Nelson and Weiss, 2008; Yoshino *et al*, 2013b). Many genome-wide miRNA expression signatures, including deep-sequencing analysis, have revealed aberrantly upregulated or downregulated expression of miRNAs in BC (Han *et al*, 2011; Chen *et al*, 2013). Subsequently, many studies have demonstrated that aberrantly downregulated miRNAs can function as tumour suppressors by regulating different target genes and cancer pathways. Therefore, identification of tumour-suppressive miRNAs and the molecular pathways mediated by these miRNAs is important to improve our understanding of cancer mechanisms.

In general, the guide-strand RNA from duplex miRNA is retained for direct recruitment of the RISC to target mRNAs, whereas the passenger-strand RNA is degraded (Chendrimada *et al*, 2007; Carthew and Sontheimer, 2009). In our previous study of PCR-based miRNA screening in BC, only *miR-199a-5p* and *miR-199b-5p* were indicated as downregulated miRNAs (Yoshino *et al*, 2011). However, in the present study, deep-sequencing analysis showed that all *miR-199* family members (*miR-199a-3p/-5p* and *miR-199b-3p/-5p*) were downregulated in BC tissues in comparison with normal bladder tissues. In addition, in our other studies of deep-sequencing analysis, we also found that some passenger strands were not degraded and acted as tumour suppressors (Matsushita *et al*, 2016; Yonemori *et al*, 2016). Thus, deep-sequencing analysis seemed to be superior to array- and

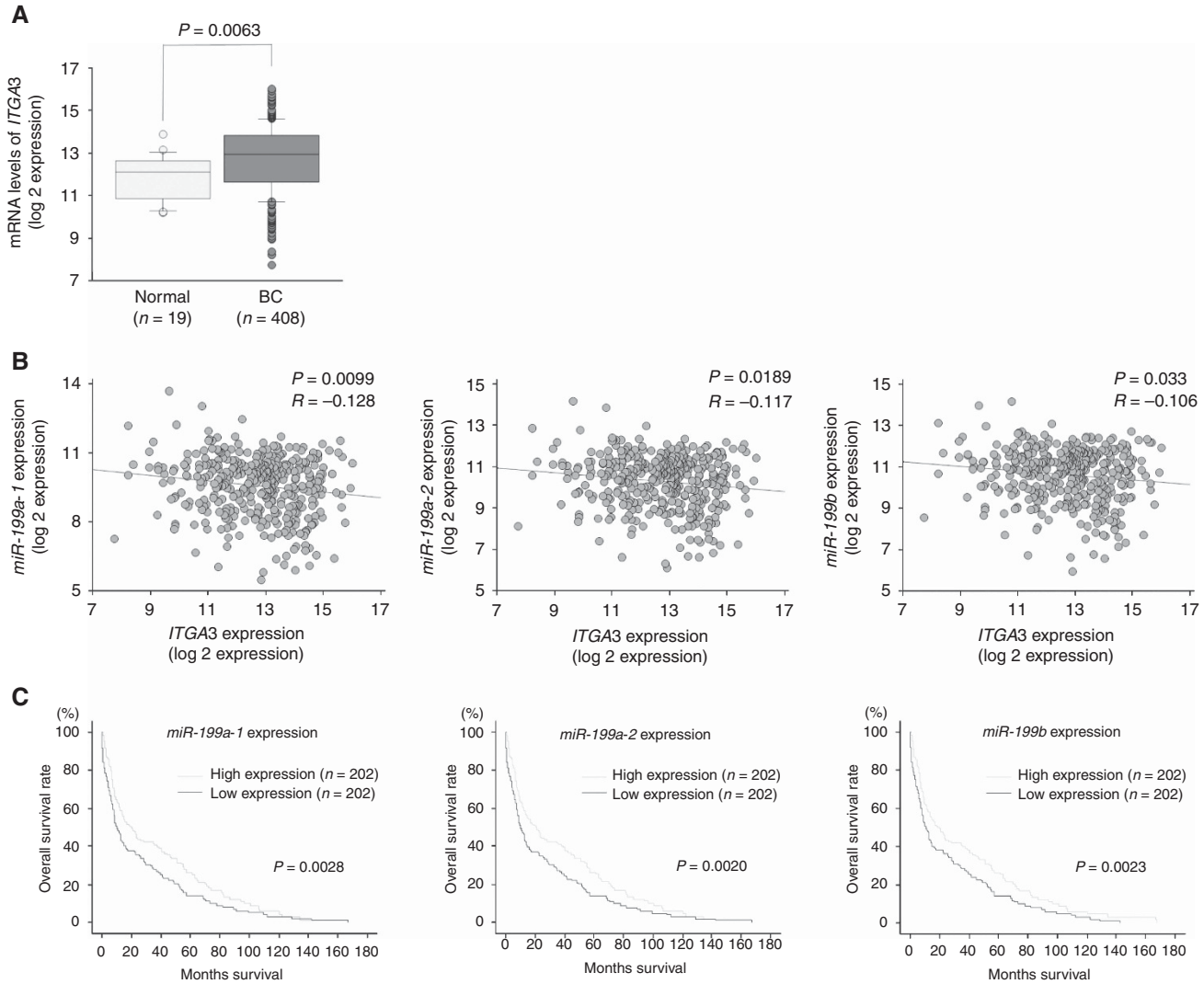

Figure 6. Analysis of the clinical BC specimens in TCGA data sets. (**A**) The expression level of *ITGA3* mRNA was significantly upregulated in BC tissues compared with that in normal bladder tissues ($P = 0.0063$). (**B**) Negative correlations between *ITGA3* expression and *miR-199a-1*, *miR-199a-2*, and *miR-199b* expression in BC tissues ($P = 0.0099$, $R = -0.128$; $P = 0.0189$, $R = -0.117$; and $P = 0.033$, $R = -0.106$, respectively). (**C**) Overall survival was significantly prolonged in patients with high *miR-199a-1*, *miR-199a-2*, and *miR-199b* expression compared with that in patients with low expression of these miRNAs ($P = 0.0028$, $P = 0.0020$, and $P = 0.0023$, respectively).

PCR-based methods and is likely to become the gold standard for comprehensive miRNA analysis in cancer genomics (Enokida *et al*, 2016). The molecular mechanism of how *miR-199* family members are downregulated in BC remains unclear. However, a recent study demonstrated that the RNA-binding protein HuR, also known as *ELAVL1* suppresses the maturation of *miR-199a* in hepatocellular carcinoma (Zhang *et al*, 2015). *ELAVL1* mRNA expression level was significantly upregulated in BC samples compared with normal bladder samples in TCGA database (Supplementary Figure 3A). Moreover, there was a significant negative correlation between *miR-199* family and *ELAVL1* expression (Supplementary Figure 3B). These data suggested that *ELAVL1* expression might be responsible for the downregulation of *miR-199* family in BC. Further studies are necessary to determine this mechanism.

miR-199 family members have been shown to have strong anticancer effects in several types of cancer, including breast cancer, colorectal cancer, and thyroid cancer, through the regulation of oncogene (Hu *et al*, 2014; Li *et al*, 2015; Sun *et al*, 2016); however, few studies have examined these miRNAs in BC. Some few previous studies have suggested that *miR-199a-5p* functions as a tumour suppressor in BC. Song *et al* (2015) reported that *miR-199a-5p* functions as a tumour suppressor by regulating MLK3/NF-κB pathway. However, the roles of *miR-199a-3p* and *miR-199b-3p/-5p* in BC remain unknown. Thus, in this study, we revealed, for the first time, that *miR-199* family members (*miR-199a-3p/-5p* and *miR-199b-3p/-5p*) functioned as tumour-suppressive miRNAs in BC. Interestingly, several previous reports showed that elevated *miR-199a* levels were associated with improved survival in patients with diffuse large B-cell lymphoma (Troppan *et al*, 2015) and patients with breast cancer (Li *et al*, 2016b). Furthermore, another report demonstrated that a panel of eight miRNAs, including *miR-199a*, may be a useful biomarker for predicting survival in BC (Zhou *et al*, 2015). Notably, in our present study using the TCGA data set, we revealed that patients with low expression of *miR-199a* or *miR-199b* exhibited poorer overall survival than patients with high expression of these miRNAs. Therefore, we speculated that these miRNAs may be effective prognostic markers in patients with BC. However, despite the observed significant correlations between *miR-199* family members and survival, our data did not reveal any significant correlations between *ITGA3* and survival. This discrepant

observation may be explained by the fact that *miR-199* family members have many target genes that influence patient prognosis in addition to *ITGA3*. Therefore, there may not be any correlations between *ITGA3* expression and survival in patients with BC. Future studies are needed to identify other target genes of *miR-199* family members.

We performed *in silico* analysis and finally obtained 10 candidate target genes of *miR-199* family. *E2F3* was at the top of the list (Supplementary Table 4), and TCGA database also showed higher *E2F3* mRNA expression in BC samples than that of normal bladder samples (Supplementary Figure 4A). In general, *E2F3* is critical for G1/S transition and has major roles in regulating tumour cell proliferation (Humbert *et al*, 2000). However, in this study, we could not find strong efficacy of inhibiting cell proliferation in the *miR-199s* transfectants as compared with that of inhibiting cell migration or invasion. In terms of *KLK3* and *SLC2A1*, TCGA database showed no significant differences in their mRNA expressions between BC and normal bladder samples. (Supplementary Figures 4B and C). Therefore, we focused on *ITGA3* as the candidate target of *miR-199* family even though it was the fourth position on the list.

Nonetheless, our findings also demonstrated that *ITGA3* has important roles in BC cell progression and may be a molecular target for BC treatment. Integrins are heterodimeric transmembrane receptors that mediate adhesion to the extracellular matrix (ECM) and immunoglobulin superfamily molecules (Desgrosellier and Cheresh, 2010). Integrin heterodimers are formed by the combination of α- and β-subunit. Many different integrin heterodimers can be expressed on a single cell, and each can interact with multiple intracellular signalling cascades (Goodman and Picard, 2012). Integrin $\alpha3$ and $\beta1$-subunits have been identified as laminin-binding receptors, for example, laminin-5 and laminin-10/-11 (Kreidberg *et al*, 1996). Extracellular matrix proteins, such as collagen, laminin, and fibronectin, regulate tissue homeostasis, organ development, inflammation, and diseases, such as cancer (Miyamoto *et al*, 2016). Integrins are essential for cell migration and invasion, because they directly mediate adhesion to these ECM proteins (Hood and Cheresh, 2002). Therefore, aberrant expression of *ITGA3* results in promoting cancer cell migration and invasion. The Cancer Genome Atlas database analysis revealed that *ITGA3* mRNA expression in BC was significantly upregulated. However, mRNA expression of *integrin β1 (ITGB1)* that compose integrin $\alpha3\beta1$ heterodimer with *ITGA3* was not significantly changed between BCs and normal bladder tissues (Supplementary Figure 4D). Our speculation is that *ITGA3* composes integrin $\alpha3\beta1$ heterodimer only with *ITGB1*. On the other hand, *ITGB1* composes many integrin heterodimers with integrin $\alpha1$–2, $\alpha4$–11, and αV as well as *ITGA3* (Jin and Varner, 2004). The Cancer Genome Atlas database revealed that the mRNA expression levels of these integrin α-subunits, except for *ITGA3*, were either not significantly changed or significantly downregulated in BCs compared with normal bladder tissues (Supplementary Figure 5). These facts imply that total expression of *ITGB1* in BC cells might balance out because of the low expression of other integrin α-subunits that bind to *ITGB1*. Overexpression of *ITGA3* has been reported in various human cancers, such as prostate cancer, breast cancer, glioma, and oral squamous cell carcinoma (Ghosh *et al*, 2006; Nakada *et al*, 2013; Shirakihara *et al*, 2013; Kurozumi *et al*, 2016). Ghosh *et al* (2006) showed that decreased integrin $\alpha3\beta1$ expression was consistent with lower invasive tumour behaviour. Moreover, several studies have shown that *ITGA3* is involved in cancer progression, cell migration, metastasis, and invasiveness in BC (Litynska *et al*, 2002; Mitsuzuka *et al*, 2005; Pochec *et al*, 2006; Behnsawy *et al*, 2011; Li *et al*, 2014, 2016a). Li *et al* (2014, 2016a) reported that aberrantly glycosylated integrin $\alpha3\beta1$ is a new biomarker of BC and that a monoclonal antibody against this integrin heterodimer has potent antitumour activities in BC. Additionally, *ITGA3* has been suggested to have a major role in modulation of other integrin receptors in BC (Litynska *et al*, 2002). Interestingly, another report showed that *ITGA3* expression was significantly associated with the incidence of intravesical recurrence after TURBT (Behnsawy *et al*, 2011). A recent report showed that tumour exosome integrins may be good biomarkers to predict organ-specific cancer metastasis (Hoshino *et al*, 2015). Thus, further studies are needed to determine the cancer molecular networks associated with integrins.

CONCLUSIONS

Downregulation of dual-stranded *miR-199* family members (*miR-199a-3p/-5p* and *miR-199b-3p/-5p*) was frequently observed in BC cells, and these miRNAs significantly inhibited cancer cell migration and invasion. To the best of our knowledge, this is the first report demonstrating that tumour-suppressive *miR-199a-3p/-5p* and *miR-199b-3p/-5p* directly regulated *ITGA3* and may be good prognostic markers for survival in patients with BC. The discovery of molecular targets mediated by tumour-suppressive miRNAs provides important insights into potential mechanisms, new therapeutics, and new biomarkers in BC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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