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# Tumour-suppressive *microRNA-144-5p* directly targets *CCNE1/2* as potential prognostic markers in bladder cancer

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**Background:** Analysis of a microRNA (miRNA) expression signature of bladder cancer (BC) by deep-sequencing revealed that clustered miRNAs *microRNA (miR)-451a*, *miR-144-3p*, and *miR-144-5p* were significantly downregulated in BC tissues. We hypothesised that these miRNAs function as tumour suppressors in BC. The aim of this study was to investigate the functional roles of these miRNAs and their modulation of cancer networks in BC cells.

**Methods:** The functional studies of BC cells were performed using transfection of mature miRNAs. Genome-wide gene expression analysis, *in silico* analysis, and dual-luciferase reporter assays were applied to identify miRNA targets. The association between *miR-144-5p* levels and expression of the target genes was determined, and overall patient survival as a function of target gene expression was estimated by the Kaplan–Meier method.

**Results:** Gain-of-function studies showed that *miR-144-5p* significantly inhibited cell proliferation by BC cells. Four cell cycle-related genes (*CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1*) were identified as direct targets of *miR-144-5p*. The patients with high *CCNE1* or *CCNE2* expression had lower overall survival probabilities than those with low expression ( $P=0.025$  and  $P=0.032$ ).

**Conclusion:** *miR-144-5p* functions as tumour suppressor in BC cells. *CCNE1* and *CCNE2* were directly regulated by *miR-144-5p* and might be good prognostic markers for survival of BC patients.

Bladder cancer (BC) is the fifth most commonly diagnosed cancer and the eighth most common cause of death in cancer patients in the 40 countries of the European Union (Ferlay *et al*, 2013). BC can be classified into two categories: non-muscle-invasive BC (NMIBC), and muscle-invasive BC (MIBC). Most BC patients (70–80%) are diagnosed with NMIBC, and recurrence rates are high (50–70%) in this group. Moreover, 15% of recurrent bladder tumours progress to MIBC. The 5-year survival rate for patients with NMIBC is close to 90%, whereas that for patients with MIBC is only approximately 60%. Furthermore, nearly 80% of patients with lymph node metastases die in the first 5 years after diagnosis

(Meeks *et al*, 2012). The molecular mechanisms of recurrence and muscle invasion are not well understood. Patients with advanced bladder cancer (with or without metastases) are generally treated with combination chemotherapy consisting of gemcitabine and cisplatin, but progression-free survival is of limited duration.

A substantial amount of evidence has suggested that microRNAs (miRNAs) are aberrantly expressed in many human cancers and have significant roles in human oncogenesis and metastasis (Di Leva and Croce, 2010). Our recent study of BC miRNA signatures by deep-sequencing revealed that 60 miRNAs were significantly downregulated in BC tissues (Itesako *et al*, 2014),

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suggesting that these miRNAs were potential candidates for tumour-suppressive miRNAs. Using our previous PCR-based BC signatures and present deep-sequence signature, we have focussed on downregulated clustered miRNAs and sequentially investigated their functional significance. Thus far, we have shown that *microRNA (miR)-1/133a*, *miR-195/497*, and *miR-23b/27b* clustered miRNAs function as tumour suppressors through their targeting of several oncogenic genes in BC cells (Yoshino *et al*, 2011; Itesako *et al*, 2014; Chiyomaru *et al*, 2015).

Deep-sequencing-based BC signatures revealed that *miR-451a*, *miR-144-3p*, and *miR-144-5p* clustered miRNAs were down-regulated in BC tissues. The aim of the present study was to investigate the functional significance of *miR-451a/144-3p/144-5p* and to identify the molecular targets regulated by these miRNAs in BC cells. Our data demonstrated that restoration of *miR-144-5p* significantly inhibited cancer cell proliferation through their targeting of oncogenic genes (*CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1*) that promoted progress through the cell cycle. The discovery of molecular targets mediated by tumour-suppressive miRNAs provides important insights into the potential mechanisms of BC oncogenesis and suggests novel therapeutic strategies and tumour markers for the treatment of BC.

## MATERIALS AND METHODS

**Clinical specimens and cell culture.** The tissue specimens for quantitative real-time reverse transcription PCRs (qRT-PCRs) were collected from BC patients ( $n=60$ ) who had received cystectomy ( $n=10$ ) or transurethral resection of their bladder tumours ( $n=50$ ) at Kagoshima University Hospital between 2003 and 2013. Normal bladder epithelia ( $n=22$ ) 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 (LH *et al*, 2009). Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were obtained from all patients. Patient details and clinicopathological characteristics are listed in Supplementary Table S1.

We used two human BC cell lines: T24, which was invasive and obtained from the American Type Culture Collection (Manassas, VA, USA); and BOY, which was established in our laboratory from an Asian male patient, aged 66 years, who was diagnosed with stage III BC with lung metastasis (Takemoto *et al*, 1997). These cell lines were maintained in minimum essential medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C (Inoguchi *et al*, 2014; Itesako *et al*, 2014).

**Tissue collection and RNA extraction.** Tissues were immersed in RNAlater (Ambion, Austin, TX, USA) and stored at –20 °C until RNA extraction was conducted. Total RNA, including miRNA, was extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. The integrity of the RNA was checked with an RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol.

**Quantitative real-time reverse transcription PCR.** Stem-loop RT-PCR (TaqMan MicroRNA Assays; P/N: 001141 for *miR-451a*, P/N: 002676 for *miR-144-3p*, and P/N: 002148 for *miR-144-5p*; Applied Biosystems, Foster City, CA, USA) was used to quantify miRNAs according to previously published conditions (Ichimi *et al*, 2009). TaqMan probes and primers for *cyclin E1*: *CCNE1* (P/N: Hs 01026536\_m1; Applied Biosystems), *cyclin E2*: *CCNE2* (P/N: Hs00180319\_m1), *cell division cycle 25A*: *CDC25A* (P/N: Hs00947994\_m1), and *protein kinase, membrane-associated tyrosine/threonine 1*: *PKMYT1* (P/N: Hs00993620\_m1) were assay-

on-demand gene expression products. We used human *GUSB* (P/N: Hs99999908\_m1) and *RNU48* (P/N: 001006), respectively, as internal controls, and the  $\Delta\Delta C_t$  method was employed to calculate the fold changes.

**Transfection with mature miRNA.** As described elsewhere (Chiyomaru *et al*, 2010), T24 and BOY cells were transfected with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) with 10 nM mature miRNA. Mature miRNAs and negative-control miRNA (Applied Biosystems) were used in gain-of-function experiments.

**Cell proliferation and flow cytometry assays.** Cell proliferation was determined with an XTT assay (Roche Applied Sciences, Tokyo, Japan) that was performed as described previously (Chiyomaru *et al*, 2010; Itesako *et al*, 2014).

BC cell lines were transiently transfected with transfection reagent only (mock), miR-control, or *miR-144-5p* in six-well tissue culture plates, as described earlier (Inoguchi *et al*, 2014). Cells were harvested by trypsinisation 72 h after transfection and washed in cold phosphate-buffered saline. For the cell cycle analysis, cells were stained with PI using the Cycletest PLUS DNA Reagent Kit (BD Biosciences, Bedford, MA, USA) following the protocol and analysed by CyAn ADP analyser (Beckman Coulter, Brea, CA, USA). The percentages of the cells in the G0/G1, S, and G2/M phases were determined and compared. Experiments were performed in triplicate.

**Genome-wide gene expression and *in silico* analysis for the identification of genes regulated by *miR-144-5p*.** To identify target genes of *miR-144-5p*, we used genome-wide gene expression analysis and *in silico* analysis. We attempted to identify *miR-144-5p* target genes using *miR-144-5p*-transfected BC cell line T24. A SurePrint G3 Human GE 8 × 60 K Microarray (Agilent) was used for expression profiling of *miR-144-5p* transfectants. The current microarray data were deposited by the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) and were assigned GEO accession number GSE66498. Gene expression data were adapted to Kyoto Encyclopaedia of Genes and Genomes pathway categories by the GENECODIS program (<http://genecodis.dacya.ucm.es>). Expression data from BC specimens used publicly available gene expression data sets in the GEO database (accession number: GSE11783 + GSE31684). The data were normalized and analysed with the GeneSpring software (Agilent) as described previously (Chiyomaru *et al*, 2015). We merged these data sets and selected putative *miR-144-5p* target genes using microRNA.org (August, 2010 release, <http://www.microrna.org>). The strategy for investigation of the target genes is shown in Supplementary Figure S1.

**Plasmid construction and dual-luciferase reporter assays.** Partial wild-type sequences of the 3'-untranslated regions (UTR) of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* or those with a deleted *miR-144-5p* target site (positions 91–96 of *CCNE1* 3'-UTR, 843–848 of *CCNE2* 3'-UTR, 775–780 of *CDC25A* 3'-UTR, and 14–19 of *PKMYT1* 3'-UTR) were inserted between the XhoI and PmeI restriction sites in the 3'-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for dual-luciferase reporter assay was described previously (Inoguchi *et al*, 2014; Itesako *et al*, 2014).

**Statistical analysis.** Relationships between two or three variables and numerical values were analysed using the Mann–Whitney *U*-test or Bonferroni-adjusted Mann–Whitney *U*-test. Spearman's rank test was used to evaluate the correlation between the expression levels of *miR-144-5p* and its target genes. We estimated overall survival in 60 BC patients by using the Kaplan–Meier method. Patients were divided into two groups according to the median value of *CCNE1* and *CCNE2* expression, and the

differences between the two groups were evaluated by the log-rank tests. We used the Expert Stat View software, version 4 (Cary, NC, USA), for these analyses.

## RESULTS

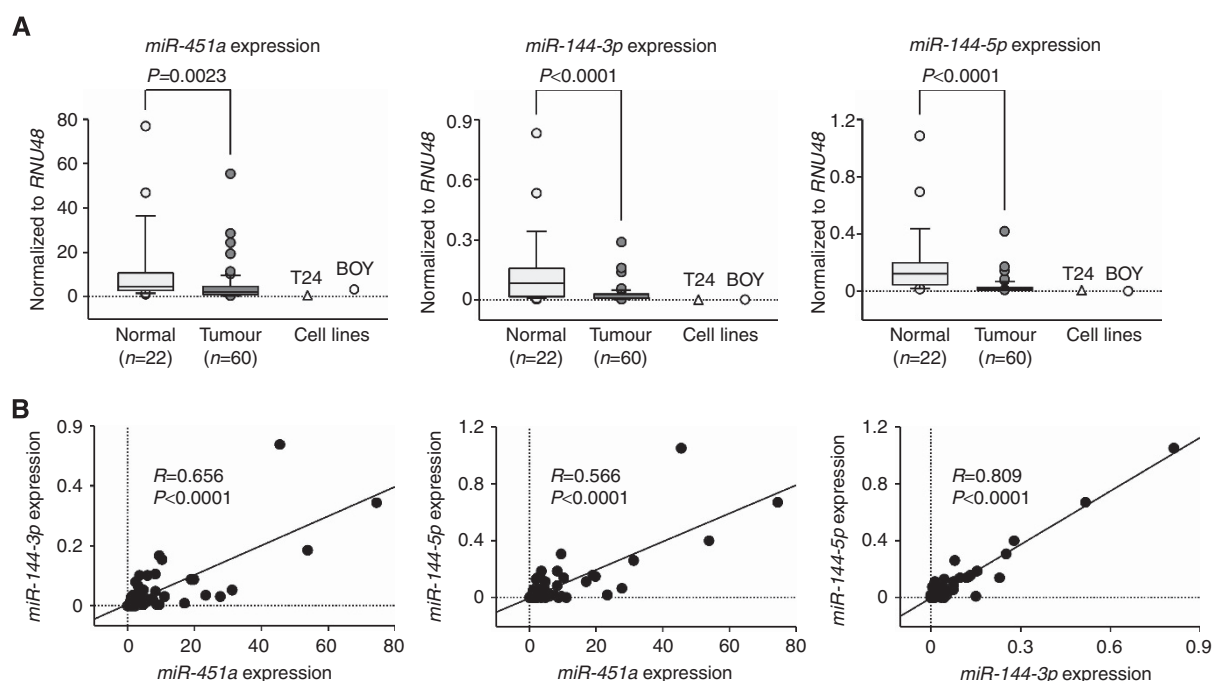
**The expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p*.** We evaluated the expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p* in BC tissues ( $n = 60$ ), normal bladder epithelia ( $n = 22$ ), and BC cell lines (T24 and BOY). The expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p* were significantly reduced in tumour tissues and BC cell lines compared with normal bladder epithelia (Figure 1A). The expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p* were analysed for their correlation with one another. Correlation coefficients of 0.656, 0.566, and 0.809 with  $P < 0.0001$  indicated that their expression levels were highly correlated with each other (Figure 1B). To avoid incidental results caused by outliers, we have excluded over  $\pm 1.0$  s.d. samples and found that there still remained significant differences in the expression levels of the miRNAs between tumour tissues and normal bladder epithelia (Supplementary Figure S2A). Similarly, there still remained significant positive correlations between the expression levels of the miRNAs (Supplementary Figure S2B). On the other hand, there were no significant relationships between any of the clinicopathological parameters (i.e., tumour stage, grade, infiltration, or survival rate) and the expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p* (data not shown).

**Effect of *miR-144-5p* transfection on cell growth and cell cycle.** We performed gain-of-function studies using the transfectants of those miRNAs to investigate the functional roles of *miR-451a*, *miR-144-3p*, and *miR-144-5p*. The XTT assay showed that *miR-144-3p* and *miR-144-5p* transfectants significantly inhibited cancer cell proliferation comparison with mock or miR-control transfectants ( $P < 0.0001$ , Figure 2A).

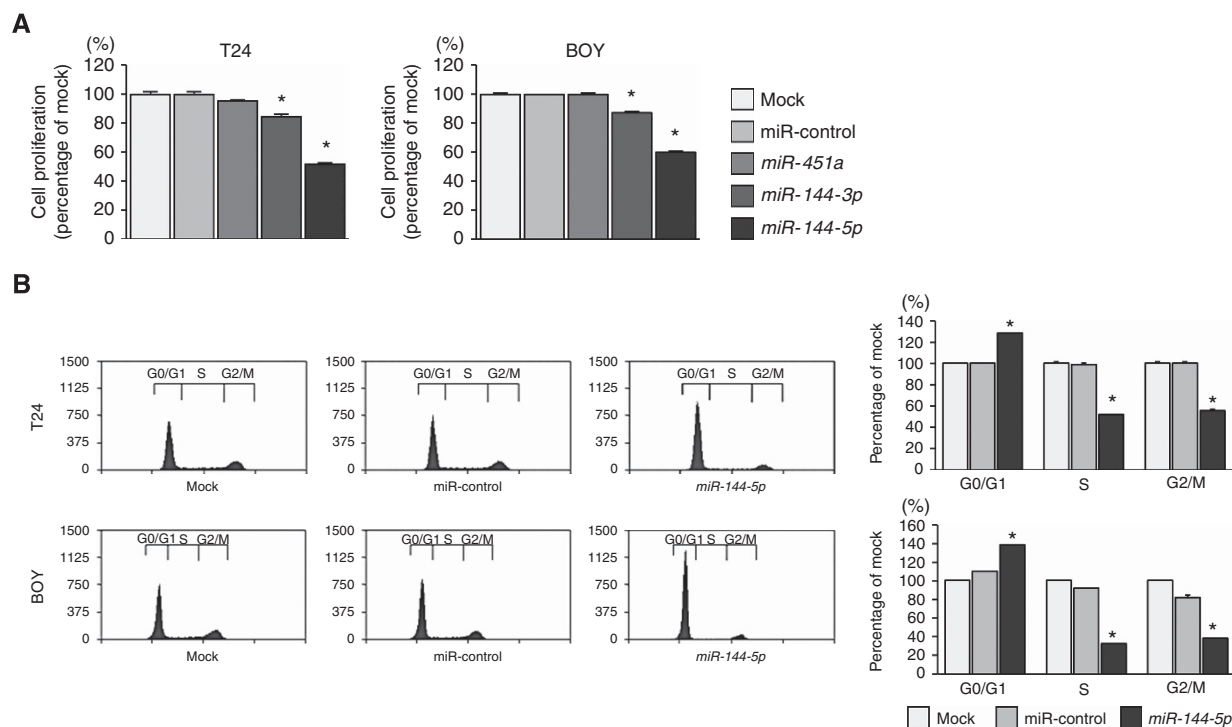
Restoration of *miR-144-5p* significantly reduced cell proliferation in T24 and BOY cells, therefore we hypothesised that *miR-144-5p* induced cell cycle arrest in BC. With regard to the cell cycle distribution, the fraction of cells in the G0/G1 phase was significantly larger in *miR-144-5p* transfectants in comparison with mock or miR-control transfectants ( $P < 0.0001$ , Figure 2B). These results suggested that *miR-144-5p* restoration induced G0/G1 arrest in BC cells.

**Identification of molecular pathways modulated by *miR-144-5p* and putative target genes in BC cells.** To gain further insight into molecular mechanisms and pathways regulated by tumour-suppressive *miR-144-5p* in BC cells, we performed genome-wide gene expression analysis using *miR-144-5p*-transfected cells, *miR-144-5p*-T24. In our microarray, a total of 1196 genes were downregulated  $< -1.0$  ( $\log_2$  ratio) in *miR-144-5p*-T24 transfectants, compared with negative control cells. The GENECODIS analysis categorised 75 significantly enriched signalling pathways (Supplementary Table S2). We focussed on the 'cell cycle' pathway and the 23 genes listed within this pathway. Of those 23 genes, 15 genes were upregulated in the GEO database, and of those 15 genes, four genes (*CCNE2*, *PKMYT1*, *CDC25A*, and *CCNE1*) contained putative binding sites for *miR-144-5p* in their 3'-UTR according to the microRNA.org database (Table 1). Our strategy for this selection of *miR-144-5p* target genes is shown in Supplementary Figure S1. The details of the top five enriched pathways excluding 'cell cycle' such as 'DNA replication', 'p53 signalling pathway', 'pathways in cancer', and 'peroxisome' are shown in Supplementary Table S3.

***CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* were directly regulated by *miR-144-5p*.** We performed qRT-PCR to confirm that restoration of *miR-144-5p* resulted in the downregulated expression of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* in T24 and BOY cells. The mRNA levels of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* were significantly reduced in *miR-144-5p* transfectants in comparison with mock or miR-control transfectants ( $P < 0.005$ , Figure 3A).



**Figure 1.** The expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p*. (A) qRT-PCR showed that the expression levels of *miR-451a*, *miR-144-3p*, and *miR-144-5p* were significantly lower in BC tissues and BC cell lines (T24 and BOY) than in non-BC tissues. (B) The correlated expression of *miR-451a*, *miR-144-3p*, and *miR-144-5p*. The correlation coefficients 0.656, 0.566, and 0.809 with  $P < 0.0001$  indicated that *miR-451a*, *miR-144-3p*, and *miR-144-5p* expression levels were highly correlated with each other.



**Figure 2.** Effects of *miR-451a*, *miR-144-3p*, and *miR-144-5p* transfection on the functionality of BC cell lines. **(A)** The XTT assay showed significant inhibition of cell proliferation in *miR-144-3p* and *miR-144-5p* transfectants in comparison with mock or *miR-control* transfectants. \* $P < 0.0001$ . **(B)** Flow cytometric analysis of cell cycle phase distribution in mock, *miR-control*, or *miR-144-5p* transfectants. The bar charts represent the percentage of mock in G0/G1, S, and G2/M phases. In *miR-144-5p* transfectants, the fraction of cells in the G0/G1 phase was significantly larger compared with mock or *miR-control* transfectants. \* $P < 0.0001$ .

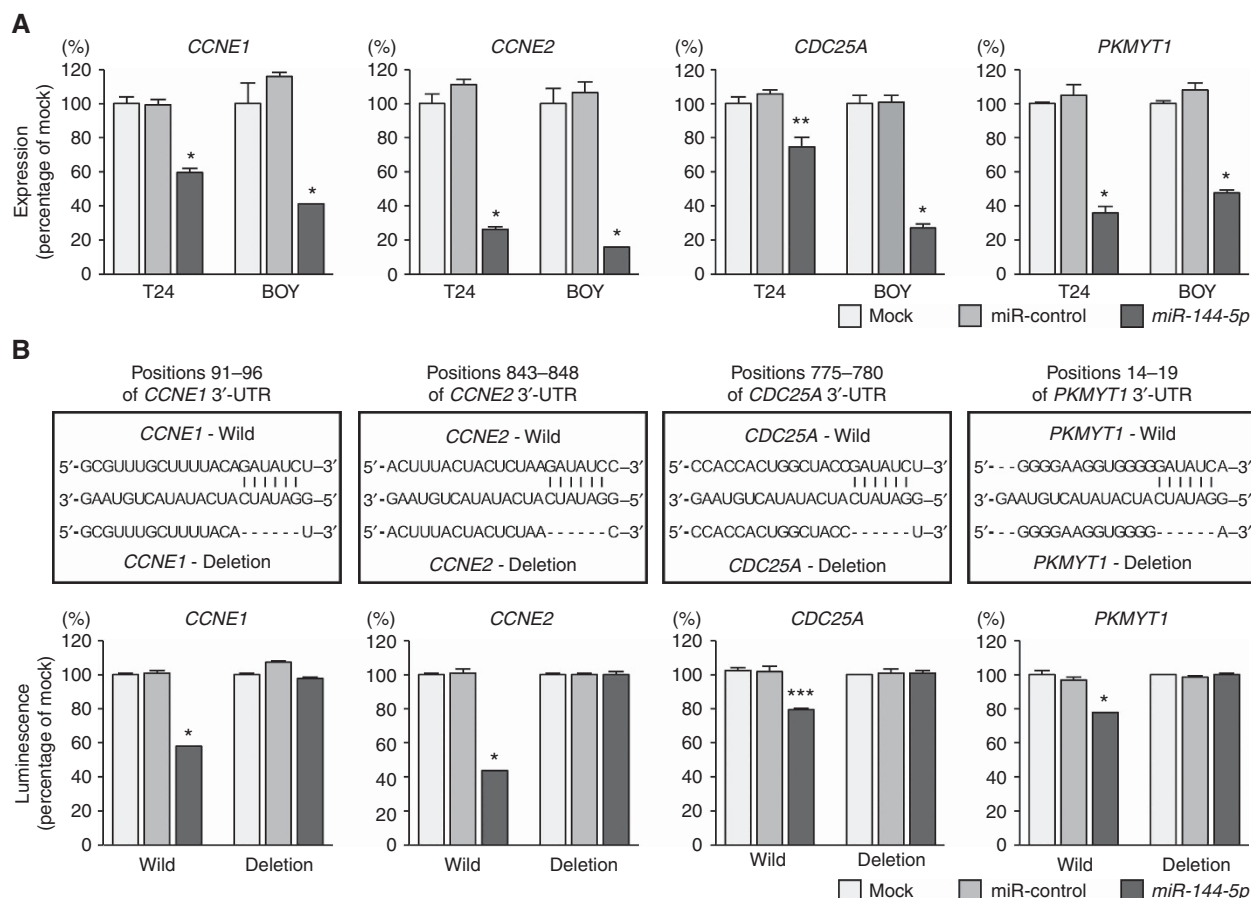
**Table 1.** Upregulated genes involved in 'cell cycle'

| Entrez gene ID | Gene symbol   | Description  | Gene expression omnibus       |          |             |
|----------------|---------------|--|-------------------------------|----------|-------------|
|                |               |  | Fold change ( $\log_2$ ratio) | P-value  | Target site |
| 991            | <i>CDC20</i>  | Cell division cycle 20                                   | 4.06                          | 1.05E-03 | —           |
| 9134           | <i>CCNE2</i>  | Cyclin E2  | 3.46                          | 1.05E-03 | +           |
| 9133           | <i>CCNB2</i>  | Cyclin B2  | 3.40                          | 1.05E-03 | —           |
| 9088           | <i>PKMYT1</i> | Protein kinase, membrane-associated tyrosine/threonine 1 | 3.35                          | 1.05E-03 | +           |
| 891            | <i>CCNB1</i>  | Cyclin B1  | 3.12                          | 1.05E-03 | —           |
| 993            | <i>CDC25A</i> | Cell division cycle 25A                                  | 3.09                          | 1.05E-03 | +           |
| 890            | <i>CCNA2</i>  | Cyclin A2  | 3.07                          | 1.05E-03 | —           |
| 898            | <i>CCNE1</i>  | Cyclin E1  | 2.73                          | 1.05E-03 | +           |
| 1869           | <i>E2F1</i>   | E2F transcription factor 1                               | 2.33                          | 1.05E-03 | —           |
| 1870           | <i>E2F2</i>   | E2F transcription factor 2                               | 1.89                          | 1.05E-03 | —           |
| 4173           | <i>MCM4</i>   | Minichromosome maintenance complex component 4           | 1.84                          | 1.05E-03 | —           |
| 4998           | <i>ORC1</i>   | Origin recognition complex, subunit 1                    | 1.74                          | 1.27E-02 | —           |
| 2810           | <i>SFN</i>    | Stratifin  | 1.37                          | 5.49E-03 | —           |
| 4176           | <i>MCM7</i>   | Minichromosome maintenance complex component 7           | 1.28                          | 1.05E-03 | —           |
| 4175           | <i>MCM6</i>   | Minichromosome maintenance complex component 6           | 1.23                          | 2.94E-03 | —           |

We performed dual-luciferase reporter assays in T24 cells to determine whether the four genes were directly regulated by *miR-144-5p*. The microRNA.org database predicted that there was one binding site for *miR-144-5p* in the 3'-UTRs of *CCNE1* (positions 91–96), *CCNE2* (positions 843–848), *CDC25A* (positions 775–780), and *PKMYT1* (positions 14–19). We used vectors encoding the partial wild-type sequence of the 3'-UTR of the mRNA, including the predicted *miR-144-5p* target

sites. We found that the luminescence intensity was significantly reduced by co-transfection with *miR-144-5p* and the vector carrying the wild-type 3'-UTR, whereas transfection with the deletion vector (binding site had been removed) blocked the decrease in luminescence ( $P < 0.0005$ , Figure 3B). These data suggested that *miR-144-5p* bound directly to specific sites in the 3'-UTR of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* mRNAs.





**Figure 3.** Direct regulation of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* by *miR-144-5p*. **(A)** The expression of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* were significantly repressed in *miR-144-5p* transfectants in comparison with mock or *miR-control* transfectants. *GUSB* was used as an internal control. \* $P < 0.0001$ ; \*\* $P = 0.0022$ . **(B)** *miR-144-5p* binding sites in the 3'-UTRs of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* mRNAs. Dual-luciferase reporter assays using vectors encoding putative *miR-144-5p* 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-144-5p* and the vector carrying the wild-type, whereas transfection with the deletion vector blocked the decrease in luminescence. These data suggested that *miR-144-5p* bound directly to specific site in the 3'-UTRs of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* mRNAs. \* $P < 0.0001$ ; \*\*\* $P = 0.0002$ .

**Expression of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* in BC clinical specimens.** The qRT-PCR analysis showed that the expression levels of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* were significantly upregulated in 60 BC specimens and BC cell lines compared with 22 normal specimens ( $P < 0.0001$ , Figure 4A). Spearman's rank test showed negative correlations between the expression of *miR-144-5p* and that of *CCNE2*, *CDC25A*, and *PKMYT1* ( $R = -0.285$ ,  $-0.364$ , and  $-0.389$ , respectively,  $P < 0.05$ , Figure 4B). To determine whether the levels of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* mRNA in tumour tissues correlated with clinicopathological factors, we analysed those gene expression levels in human tumour samples. Only *CCNE1* expression increased from the NMIBC to MIBC ( $P = 0.0498$ , Supplementary Figure S3). Kaplan-Meier analysis showed that the high *CCNE1* and *CCNE2* expression groups had significantly lower overall survival probabilities compared with the low *CCNE1* and *CCNE2* expression groups ( $P = 0.0251$  and  $P = 0.0324$ , respectively, Figure 5).

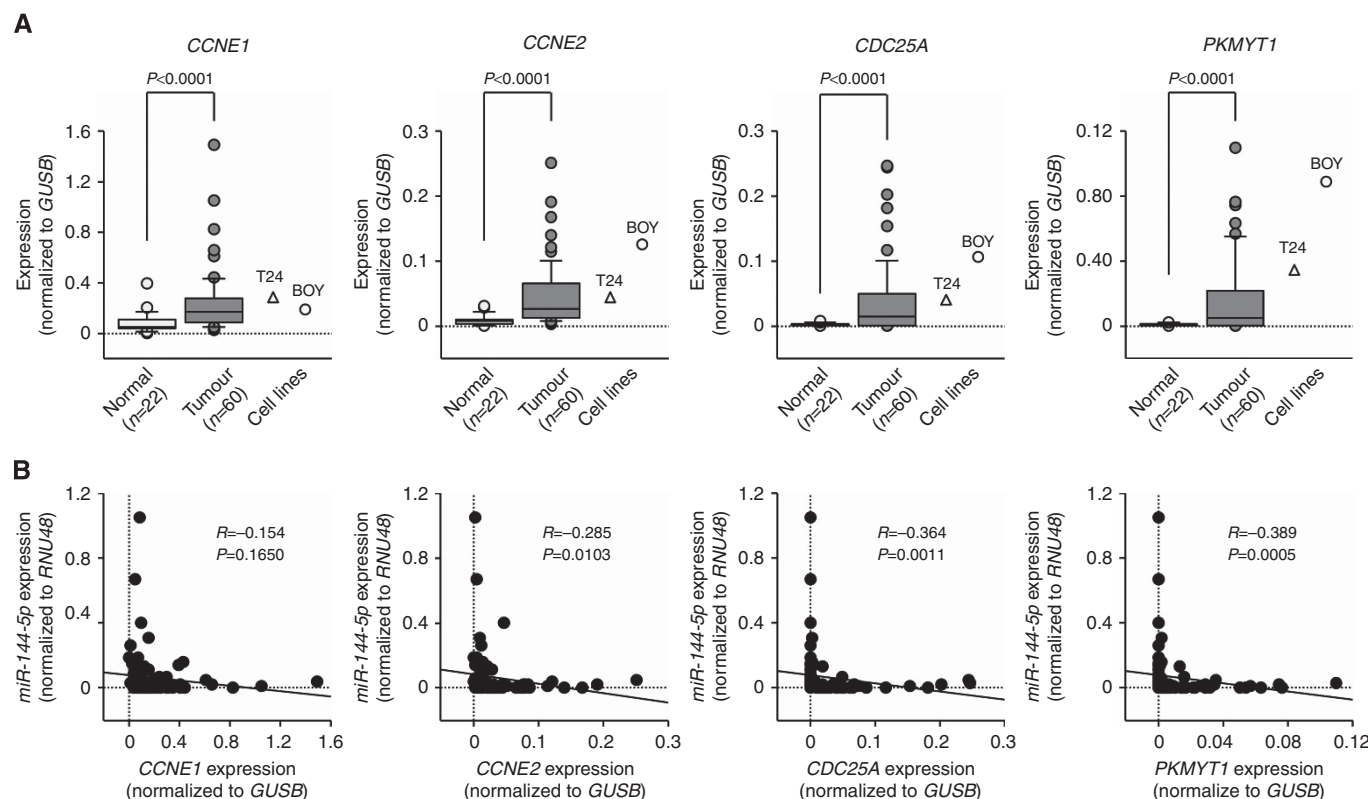
## DISCUSSION

Aberrantly expressed miRNAs are frequently annotated in BC expression signatures. We suggest that they might be driver molecules for BC development, progression, and metastasis.

Several expression signatures showed that *miR-1*, *miR-26a*, *miR-29a/c*, *miR-133a/b*, *miR-143*, *miR-145*, and *miR-195* were down-regulated in BC tissues, suggesting that these miRNAs function as tumour suppressors (Yoshino *et al.*, 2013c). Interestingly, aberrantly expressed *miR-1/133a*, *miR-29a/b*, *miR-143/145*, and *miR-195/497* are located close together in the human genome, constituting a cluster of miRNAs. Our previous studies demonstrated that *miR-1/133a*, *miR-143/145*, and *miR-195/497* clustered miRNAs functioned as tumour suppressors via their targeting of several oncogenic pathways in BC cells and other types of cancers (Nohata *et al.*, 2011, 2012; Yoshino *et al.*, 2011, 2013a; Kawakami *et al.*, 2012; Kojima *et al.*, 2012, 2014; Yamasaki *et al.*, 2012; Itesako *et al.*, 2014). Identification of tumour-suppressive miRNAs and their mediated molecular pathways adds significantly to our understanding of BC oncogenesis.

Deep-sequencing analysis seems to be superior to array-based or PCR-based methods that can only determine a limited number of known miRNAs. In the future, deep-sequencing analysis is likely to become the gold standard for comprehensive miRNA analysis in cancer genomics. Our deep-sequence-based signature showed that *miR-451a*, *miR-144-3p*, and *miR-144-5p* clustered miRNAs were downregulated in BC tissues. In the present study, downregulation of the cluster was validated in BC clinical specimens and suggested that these miRNAs function as tumour suppressors in BC cells.

In this study, we focussed on *miR-144-5p* because it showed the strongest antitumour effects in BC cells. At present, there are few



**Figure 4.** Expression levels of four *miR-144-5p* target genes. **(A)** The expression levels of *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* were significantly upregulated in BC tissues and BC cell lines in comparison with the normal bladder tissues. **(B)** The correlated expression levels between *miR-144-5p* and *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1*. Expression levels of *miR-144-5p* and *CCNE1* or *CDC25A* or *PKMYT1* were significantly negatively correlated with each other.

reports about *miR-144-5p* in human cancers. To better understand BC oncogenesis, we identified target genes and pathways of *miR-144-5p* using *in silico* analysis. Recent miRNA studies in our laboratory have utilised this strategy to identify novel molecular targets and pathways regulated by tumour-suppressive miRNAs in several cancers, including BC (Yoshino *et al*, 2013b; Fukumoto *et al*, 2014; Goto *et al*, 2014; Inoguchi *et al*, 2014; Nishikawa *et al*, 2014; Chiyomaru *et al*, 2015). Molecular target searches revealed that four cell cycle-promoting genes, *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1*, were directly targeted by *miR-144-5p* in BC cells. Overexpression of these genes was confirmed in BC clinical specimens. Furthermore, Kaplan–Meier analysis showed that high *CCNE1* and *CCNE2* expression groups had significantly lower overall survival. These results suggest that these genes might have the potential to be good prognostic markers and therapeutic targets in BC.

Cyclin E proteins have critical roles in the G1 phase and in the G1–S phase transition with cyclin-dependent kinase 2 (CDK2). Overexpression of *CCNE1* and *CCNE2* has been reported in many types of human cancers, including BC. It is well established that these genes act as oncogenes (Donnellan and Chetty, 1999). Our present data clearly showed that restoration of *miR-144-5p* function in BC cells inhibited the expression of both *CCNE1* and *CCNE2* and significantly induced G1 arrest in BC cells. In the *in vitro* studies, *CCNE/CDK2* activation has important role in tumorigenesis (Hwang and Clurman, 2005). Fu *et al* (2014) showed that overexpression of *CCNE1* was associated with aggressiveness in clinical BC specimens. In addition, *CCNE1/CDK2* inhibition significantly reduced tumour growth in trastuzumab-resistant HER2<sup>±</sup> breast cancer cell (Scaltriti *et al*, 2011). These results lead to an idea that *CCNE*-targeted therapy by *miR-144-5p* might be a new strategy in human cancers.

Our present data also showed that the *CDC25A* gene was regulated by *miR-144-5p* in BC cells. Overexpression of *CDC25A* has been reported in various cancers and contributes to cancer cell progression (Brunetto *et al*, 2013). Thus *CDC25A* is considered an oncogene, as it can cooperate with *CCNE1/2* in promoting G1/S cell cycle transition in BC cells. Direct regulation of cell cycle regulators *CCNE1/2* and *CDC25A* by tumour-suppressive *miR-144-5p* is important in developing new treatment of BC.

In previous studies of miRNA regulation of *CCNE1*, *CCNE2*, and *CDC25A* in cancers, it was shown that *CCNE1* was regulated by *miR-15* in glioma cells (Xia *et al*, 2009), *miR-7* in HCC (Zhang *et al*, 2014), *miR-132* in osteosarcoma cells (Wang *et al*, 2014), *miR-195-5p* in breast cancer (Luo *et al*, 2014), and *miR-497* in cervical carcinoma cells (Han *et al*, 2014). In contrast, *CCNE2* was regulated by *miR-26a* in pancreatic cancer (Deng *et al*, 2013), *miR-1699* in ovarian cancer cells (Lee *et al*, 2012), *miR-200* in MALT lymphoma (Cai *et al*, 2012), and *miR-449* in gastric cancer (Bou Kheir *et al*, 2011). *CDC25A* is regulated by several miRNAs, such as the *miR-195/497* cluster in HCC (Furuta *et al*, 2013), *let-7* in lung cancer cells (Johnson *et al*, 2007), and *miR-125b* in glioma cells (Shi *et al*, 2010). We have recently reported that *miR-195* and *miR-497* act as tumour suppressors in BC cells and that they target *BIRC5*, a member of the inhibitor of apoptosis family (Itesako *et al*, 2014). Interestingly, our unpublished data show that the tumour-suppressive *miR-195/497* cluster targets *CCNE1*, *CCNE2*, and *CDC25A* in BC cells (data not shown). Our tumour-suppressive miRNAs studies suggest that activation of the cell cycle and inhibition of cancer cell apoptosis might have roles in the recurrence of BC cancer. These findings suggest that molecules promoting G1/S cell cycle transition in BC cells would be critically important targets for BC therapeutic strategies.

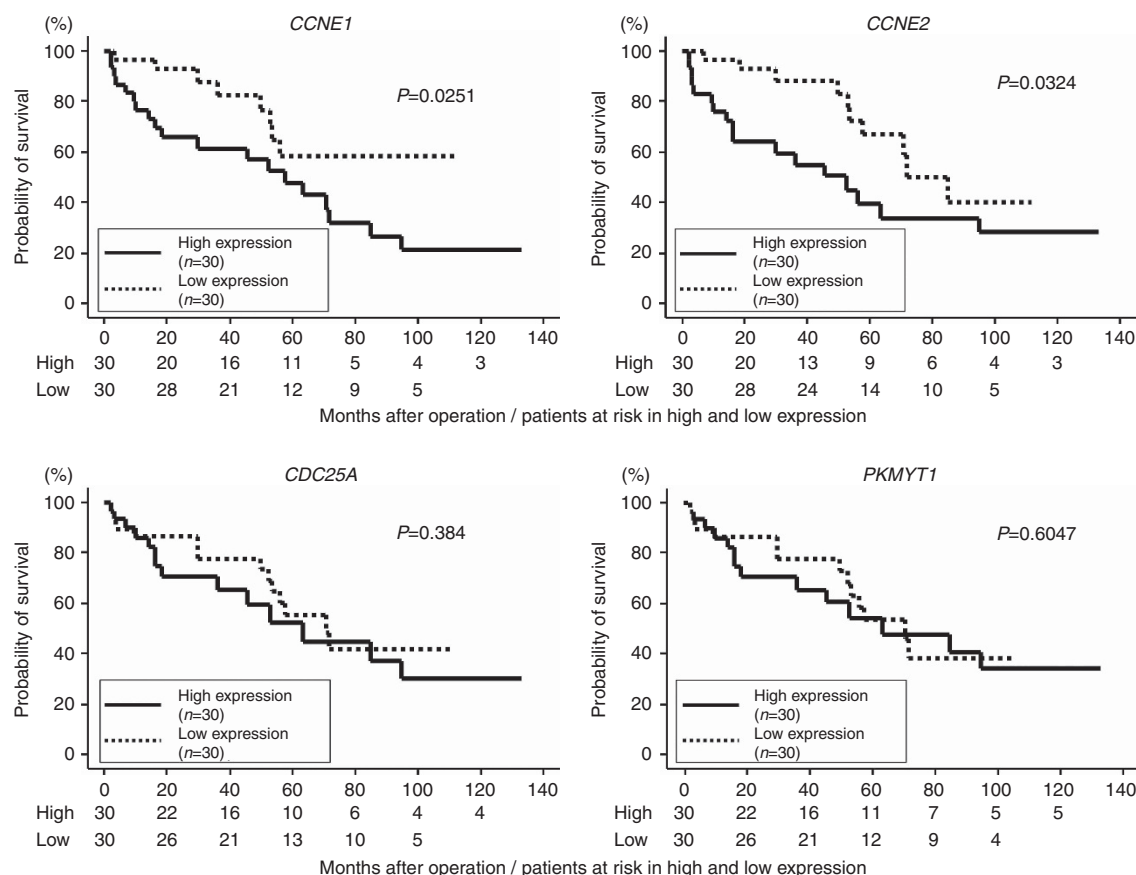


Figure 5. Kaplan–Meier survival plots for high and low expression groups for *CCNE1*, *CCNE2*, *CDC25A*, and *PKMYT1* determined for 60 patients. Overall survival was significantly prolonged in patients with low *CCNE1* and *CCNE2* expression vs patients with high expression ( $P=0.0251$  and  $P=0.0324$ , respectively).

## CONCLUSIONS

Downregulation of the *miR-451a*, *miR-144-3p*, and *miR-144-5p* cluster was frequently observed in BC cells, and *miR-144-5p* significantly inhibited cancer cell proliferation by inducing cell cycle arrest. Cell cycle regulator genes, *CCNE1*, *CCNE2*, and *CDC25A*, were directly modulated by *miR-144-5p*. Furthermore, the expression levels of *CCNE1* and *CCNE2* might be good disease prognostic markers in BC.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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