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MicroRNA expression signature of castration-resistant prostate cancer: the *microRNA-221/222* cluster functions as a tumour suppressor and disease progression marker

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Background: Our present study of the microRNA (miRNA) expression signature in castration-resistant prostate cancer (CRPC) revealed that the clustered miRNAs *microRNA-221* (*miR-221*) and *microRNA-222* (*miR-222*) are significantly downregulated in cancer tissues. The aim of this study was to investigate the functional roles of *miR-221* and *miR-222* in prostate cancer (PCa) cells.

Methods: A CRPC miRNA signature was constructed by PCR-based array methods. Functional studies of differentially expressed miRNAs were analysed using PCa cells. The association between miRNA expression and overall survival was estimated by the Kaplan–Meier method. *In silico* database and genome-wide gene expression analyses were performed to identify molecular targets regulated by the *miR-221/222* cluster.

Results: *miR-221* and *miR-222* were significantly downregulated in PCa and CRPC specimens. Kaplan–Meier survival curves showed that low expression of *miR-222* predicted a short duration of progression to CRPC. Restoration of *miR-221* or *miR-222* in cancer cells revealed that both miRNAs significantly inhibited cancer cell migration and invasion. *Ecm29* was directly regulated by the *miR-221/222* cluster in PCa cells.

Conclusions: Loss of the tumour-suppressive *miR-221/222* cluster enhanced migration and invasion in PCa cells. Our data describing targets regulated by the tumour-suppressive *miR-221/222* cluster provide insights into the mechanisms of PCa and CRPC progression.

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer-related death among men in developed countries (Siegel *et al*, 2013). Androgen signalling through the androgen receptor (AR) is an important oncogenic pathway for PCa progression. Most patients initially respond to androgen-deprivation therapy (ADT), but eventually acquire resistance and progress to castration-resistant prostate cancer (CRPC). One of the most important features of PCa is the heterogeneity of PCa cells, which results in diverse outcomes in patients with PCa, even for those in the same risk group (Scher

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et al, 1999; Colloca, 2012). Prostate-specific antigen (PSA) does not accurately reflect the progression of CRPC. Thus, effective biomarkers for follow-up and detection of CRPC are needed. Moreover, most clinical trials for advanced PCa have shown limited benefits, eventually resulting in disease progression and metastasis (Chi *et al*, 2009). Therefore, understanding the molecular mechanisms of the androgen-independent and metastatic signalling pathways underlying PCa using current genomic approaches would help to improve therapies for and prevention of the disease.

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules (18–25 bases in length) that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (Bartel, 2004). Aberrantly expressed miRNAs contribute to the initiation, development, and metastasis of several types of cancers, including PCa (Esquela-Kerscher and Slack, 2006). MicroRNAs are unique in their ability to regulate multiple protein-coding genes, and normal regulatory mechanisms can be disrupted by the aberrant expression of tumour-suppressive or oncogenic miRNAs in cancer cells. Therefore, identification of aberrantly expressed miRNAs is an important first step towards elucidating miRNA-mediated oncogenic targets.

In this study, we constructed the miRNA expression signature of CRPC using clinical specimens because the development of therapeutic strategies is a central theme in the advancement of PCa treatments. Using CRPC expression signature data, we investigated the specific roles of miRNAs in PCa and CRPC oncogenesis by examining differentially expressed miRNAs. Data from our previous PCa signature (Fuse *et al*, 2012) and present CRPC signature showed that the clustered miRNAs *miR-221* and *miR-222* were significantly downregulated in PCa and CRPC tissues, suggesting that these miRNAs may act as tumour suppressors. In contrast, previous studies have indicated that *miR-221* and *miR-222* function as oncogenes in PCa cells (Sun *et al*, 2014; Yang *et al*, 2014). Moreover, overexpression of these clustered miRNAs has been observed in breast cancer (Shah and Calin, 2011). Therefore, the functional roles of *miR-221* and *miR-222* in human cancers, including PCa, are still controversial.

The aim of this study was to investigate the functional significance of *miR-221* and *miR-222* and to identify molecular targets and pathways regulated by this miRNA cluster in PCa cells. We expect that this analysis will provide important insights into the potential molecular mechanisms of PCa oncogenesis and metastasis and will facilitate the development of novel diagnostic and therapeutic strategies for the treatment of PCa.

MATERIALS AND METHODS

Patients and clinical prostate specimens. Clinical prostate specimens were obtained from patients admitted to the Teikyo University Chiba Medical Centre Hospital from 2008 to 2013. Ninety-two patients with elevated PSA levels underwent transrectal prostate needle biopsy, and three patients who died of CRPC underwent autopsies. Prostate cancer tissues ($n = 54$), noncancerous prostate tissues (non-PCa, $n = 38$), and CRPC tissues ($n = 8$) were used in this study. The patients' backgrounds and clinicopathological characteristics are summarised in Supplementary Tables 1 and 2. Supplementary Figure 1 describes the clinical courses of the patients with CRPC. Samples were staged according to the UICC TNM classification (Edge *et al*, 2010). Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Teikyo University.

For pathological verification of tissue composition, a pair of needle biopsy specimens was collected from the same region as from patients in this study, and one was subjected to pathological verification; no cancerous tissue was found in non-PCa specimens. Castration-resistant prostate cancer was defined according to guidelines published by the European Association of Urology (Heidenreich *et al*, 2014a).

Construction of the miRNA expression signature of CRPC. miRNA expression patterns were evaluated using the TaqMan LDA Human microRNA Panel v2.0 (Applied Biosystems, Foster City, CA, USA). The assay procedure was performed as described previously (Nohata *et al*, 2011; Fuse *et al*, 2012; Fukumoto *et al*, 2014). A description of the real-time PCR assay and the list of human miRNAs included in the panel can be found on the manufacturer's website (http://www.appliedbiosystems.com). Analysis of relative miRNA expression data was performed using GeneSpring GX software version 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. A cutoff *P*-value of less than 0.05 was used to narrow down the candidates after global normalisation of the raw data. After global normalisation, additional normalisation was carried out with *U6*.

Cell culture. Human PCa cells (RWPE-1, LNCaP, C4-2, PC3, and DU145 cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP, C4-2, PC3, and DU145 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. RWPE-1 cells were cultured in keratinocyte serum-free medium containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract.

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies) as described previously (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a).

Quantitative real-time reverse transcription–PCR (RT–qPCR). The procedure for PCR quantification was carried out as previously described (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a). The expression levels of *miR-221* (Assay ID: 000524) and *miR-222* (Assay ID: 002276) were analysed by TaqMan RT–qPCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalised to *RNU48* (Assay ID: 001006). TaqMan probes and primers for *Ecm29*/*KIAA0368* (P/N: Hs00322433_m1) and *GUSB* (P/N: Hs00939627_m1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products).

Transfection with miRNA mimic and small interfering RNA (siRNA). The following miRNA mimic species were used in this study: Ambion Pre-miR miRNA precursor for *hsa-miR-221-3p (A)* (product ID: PM10337; Austin, TX, USA) and miRIDIAN miRNA Human *hsa*-miR-221-3p-Mimic for *hsa-miR-221(T)* (product ID: C-300578-05; Thermo Fisher Scientific, Waltham, MA, USA); Ambion Pre-miR miRNA precursor for *hsa-miR-222-3p (A)* (product ID: PM11376) and miRIDIAN miRNA Human *hsa*-miR-222-3p-Mimic for *hsa-miR-222(T)* (product ID: C-300579-07; Thermo Fisher Scientific). The following siRNAs were used: Stealth Select RNAi siRNA; si-*KIAA0368 (Ecm29)* (cat no. HSS146372; Invitrogen); and negative control miRNA/siRNA (P/N: AM17111, Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMAX reagent (Invitrogen). The transfection procedures and transfection efficiencies of miRNA in PC3 and DU145 cells were reported previously (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a).

Cell proliferation, migration, and invasion assays. To investigate the functional significance of the *miR-221/222* cluster or gene

silencing by siRNA knockdown, we performed cell proliferation, migration, and invasion assays using PC3 and DU145 cells, as previously described (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a).

Genome-wide gene expression and *in silico* analyses for the identification of genes regulated by the *miR-221/222* cluster. We performed a combination of *in silico* and genome-wide gene expression analyses. First, genes regulated by the *miR-221/222* cluster were listed using the TargetScan database, as described previously (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a). Next, to identify upregulated genes in PCa, we analysed a publicly available gene expression data set in GEO (accession number: GSE29079). Finally, we performed genome-wide gene expression analysis using *miR-221*- and *miR-222*-transfected PC3 and DU145 cells. A SurePrint G3 Human GE 60K Microarray (Agilent Technologies) was used for expression profiling of each miRNA transfectant in comparison with negative control miRNA transfectants. Finally, downregulated mRNAs containing *miR-221/222* target sites were listed as putative target genes of these miRNAs.

Western blotting. Immunoblotting was performed with rabbit anti-KIAA0368 (Ecm29) antibodies (1 : 1000, PA5-29467; Pierce Antibodies, Thermo Scientific, Fremont, CA, USA), and anti-GAPDH antibodies (1 : 1000, ab8245; Abcam, Cambridge, UK) were used as an internal loading control. Membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase-linked antibodies (7074; Cell Signaling Technology, Danvers, MA, USA). Complexes were visualised with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). The experimental procedures were performed as described in our previous studies (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a).

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the *KIAA0368* (*Ecm29*) 3′ untranslated region (UTR) or those with deleted *miR-221/222* target sites (position 97–103 of the *KIAA0368* 3′ UTR) were inserted between the *Xho*I–*Pme*I restriction sites in the 3′ UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The protocol for vector construction was described previously (Kinoshita *et al*, 2013; Nohata *et al*, 2013; Goto *et al*, 2014a).

Immunohistochemistry. A total of eight CRPC specimens were used (Supplementary Table 2). Tissue specimens were immunostained following the manufacturer's protocol with the Ultra-Vision Detection System (Thermo Scientific). Primary rabbit polyclonal antibodies against AR (1 : 50, ab9474; Abcam), antibodies against PSA (1 : 500, HPA000764; Sigma-Aldrich), and antibodies against Ecm29 (1 : 500, PA5-29467; Pierce Antibodies, Thermo Scientific) were used for immunochemistry. The slides were treated with biotinylated goat antibodies (Histofine SAB-PO kit; Nichirei, Tokyo, Japan).

Statistical analysis. The relationships between two groups and the numerical values obtained by RT–qPCR were analysed using Mann–Whitney *U*-tests. Spearman's rank test was used to evaluate the correlations between the expression of *miR-221* and *miR-222*. The relationships among more than three variables and numerical values were analysed using the Bonferroni-adjusted Mann–Whitney *U*-test. Survival analysis was evaluated by the Kaplan–Meier method and log-rank test. A multivariate Cox proportional hazards model was used to establish independent factors for survival. The Kaplan–Meier method and log-rank test were performed using Stat Mate software (version 4.01, ATMS Co., Tokyo, Japan); all other analyses were performed using Expert StatView (version 5, SAS Institute Inc., Cary, NC, USA).

RESULTS

Identification of downregulated miRNAs in CRPC specimens by miRNA expression signatures. We evaluated the expression levels of mature miRNAs in CRPC specimens by PCR-based array analysis. Of the eight CRPC specimens used in this study, we used six specimens (Nos. 3–8, Supplementary Table 2) for array analysis. Expression signatures revealed that 42 miRNAs were significantly downregulated in CRPC specimens compared with our previous miRNA signature of normal prostate tissue (Table 1). In addition, we compared miRNA expressions in CRPC with our previous miRNA signature of hormone naïve PCa specimens (Supplementary Table 3). We focused on downregulated miRNAs in CRPC compared with normal prostate tissue in this study. Three miRNA clusters, that is, *miR-221/222*, *miR-23b/27b/24*, and *miR-106a/20b*, were found in this signature. We focused on the *miR-221/222* cluster for further studies.

Expression levels of *miR-221* and *miR-222* in PCa specimens and cell lines. The chromosomal location of *miR-221/222* in the human genome is shown in Supplementary Figure 2. Database analysis demonstrated that these miRNAs were closely located on human chromosome Xp11.3 within 800 base pairs.

We evaluated the expression levels of the clustered miRNAs (*miR-221/222*) in non-PCa ($n = 38$), PCa ($n = 54$), and CRPC ($n = 8$) tissues. In patients from whom non-PCa tissues were collected, the median PSA level was 7.315 ng ml^{-1} (range = 4.3–35.5 ng ml^{-1}). In contrast, in patients from whom PCa tissues were collected, PSA levels were quite high, with a median of 212 ng ml^{-1} (range = 3.45–3750 ng ml^{-1}). Forty-four patients with PCa had progressive disease classified as stage IV according to TNM classification (Supplementary Table 1). The results of H&E staining and immunohistochemical staining of AR in CRPC specimens are shown in Supplementary Figure 3.

The expression levels of *miR-221* and *miR-222* were significantly downregulated ($P < 0.0001$) in cancer tissues compared with noncancerous tissues (Figures 1A and B). Furthermore, the levels of these miRNAs were significantly downregulated ($P < 0.0001$) in CRPC tissues compared with noncancerous tissues (Figures 1A and B). Spearman's rank test showed positive correlations between the expression of *miR-221* and *miR-222* ($R = 0.891$ and $P < 0.0001$; Figure 1C).

PC-3 cells had slightly higher *miR-221* expression level than LNCaP or C4–2 cell lines. DU145 cells exhibited lowest *miR-221* expression levels among these cell lines. As for *miR-222*, the expression levels were not consistent with aggressiveness of cell lines.

Associations between the expression levels of *miR-221* and *miR-222* and clinicopathological features in PCa specimens. Among 54 patients with PCa, 52 underwent ADT with luteinising hormone-releasing hormone agonist and anti-androgens (Supplementary Table 1). A total of 20 ADT-treated patients progressed to CRPC over a median follow-up of 17.2 months. The risk of progression to CRPC was then analysed in patients with high *vs* low *miR-221/222* expression. Low expression of *miR-222* was associated with shorter progression-free interval ($P = 0.0173$; Figure 1E). However, *miR-221* did not predict the time to CRPC in these PCa patients ($P = 0.147$; Figure 1D). Similarly, various clinicopathological parameters and CRPC progression-free intervals were evaluated. cT4 or cM1 was associated with shorter CRPC progression-free interval ($P = 0.0403$ and $P = 0.0309$; Supplementary Figures 4A and C). However, no significant differences in cN stage or PSA levels were observed between the two groups in this cohort (Supplementary Figures 4B and D).

Univariate and multivariate Cox proportional hazards models were used to assess independent predictors of time to progression to CRPC, including Gleason score, cT stage, cN stage, cM stage,

Table 1. Downregulated miRNAs in CRPC

miRNA	Log2 ratio (CRPC/non-PCa)	non-PCa	CRPC	*P*-value
hsa-miR-205	− 11.34	0.00318	1.228E-06	0.0401
hsa-miR-378	− 11.15	0.00336	1.479E-06	0.0485
hsa-miR-222	− 8.40	0.11068	0.0003286	0.0161
hsa-miR-143	− 8.34	0.05562	0.0001715	0.0465
hsa-miR-133a	− 7.70	0.01215	5.847E-05	0.0203
hsa-miR-23b	− 6.50	0.00103	1.138E-05	0.0276
hsa-miR-345	− 6.45	0.00266	3.035E-05	0.0264
hsa-miR-150	− 6.25	0.00945	0.0001244	0.0084
hsa-miR-139-5p	− 6.22	0.00299	4.018E-05	0.0075
hsa-miR-221	− 6.21	0.00208	2.822E-05	0.0355
hsa-miR-484	− 5.68	0.01537	0.0003003	0.0144
hsa-miR-29c	− 5.55	0.01459	0.0003123	0.0447
hsa-miR-28-3p	− 5.44	0.00672	0.0001547	0.0262
hsa-miR-27b	− 5.28	0.00134	3.444E-05	0.0362
hsa-miR-320	− 5.26	0.01826	0.0004779	0.0382
hsa-miR-30a*	− 5.21	0.00730	0.000197	0.0048
hsa-miR-532-3p	− 5.21	0.00113	3.05E-05	0.0415
hsa-miR-574-3p	− 5.15	0.02286	0.0006437	0.0048
hsa-miR-30e*	− 5.13	0.00553	0.0001576	0.0105
hsa-miR-24	− 5.06	0.14362	0.0043124	0.0215
hsa-miR-196b	− 5.01	0.00183	5.666E-05	0.0023
hsa-miR-199a-3p	− 4.50	0.01030	0.0004562	0.0434
hsa-miR-218	− 4.43	0.00137	6.354E-05	0.0233
hsa-miR-27a	− 4.31	0.00235	0.0001182	0.0426
hsa-miR-152	− 4.25	0.00175	9.14E-05	0.0341
hsa-miR-660	− 3.96	0.00165	0.0001059	0.0396
hsa-miR-126*	− 3.61	0.00169	0.0001386	0.0191
hsa-miR-146a	− 3.55	0.00718	0.0006132	0.0099
hsa-miR-20b	− 3.52	0.00430	0.0003738	0.0333
hsa-miR-193b	− 3.38	0.00966	0.0009259	0.0213
hsa-miR-106a	− 2.93	0.03526	0.00462	0.0359
hsa-miR-125a-5p	− 2.86	0.00179	0.0002467	0.0062
hsa-miR-149	− 2.81	0.00538	0.000767	0.0211
hsa-miR-17	− 2.77	0.02746	0.0040348	0.0460
hsa-miR-223	− 2.66	0.01701	0.0026952	0.0189
hsa-miR-454	− 2.46	0.00136	0.0002463	0.0077
hsa-miR-186	− 2.46	0.00464	0.0008433	0.0093
hsa-miR-151-3p	− 2.34	0.00238	0.0004702	0.0353
hsa-miR-16	− 2.07	0.05451	0.0129807	0.0193
hsa-miR-342-3p	− 2.04	0.00623	0.0015125	0.0207
hsa-miR-200c	− 1.58	0.03969	0.0132931	0.0324
hsa-miR-126	− 1.22	0.04305	0.0184953	0.0434

Abbreviations: CRPC = castration-resistant prostate cancer; miRNA = microRNA; PCa = prostate cancer.

PSA, age, and *miR-222* expression. *miR-222* expression was a prognostic factor of patient outcomes for PCa patients treated with ADT both in the univariate and multivariate analyses (univariate: hazard ratio = 0.352, 95% confidence interval = 0.135–0.917, $P = 0.0326$; multivariate: hazard ratio = 0.206, 95% confidence interval = 0.067–0.637, $P = 0.0060$; Table 2).

Effects of restoring *miR-221* or *miR-222* expression on cell proliferation, migration, and invasion in PC3 and DU145 cells. To investigate the functional roles of the *miR-221/222* cluster, we performed gain-of-function studies using miRNA transfection in PC3 and DU145 cells. We used two sources of *miR-221* and *miR-222* mimic (*miR-221*-A and *miR-222*-A: Ambion; *miR-221*-T and *miR-222*-T: Thermo Scientific Dharmacon, Waltham, MA, USA) to ensure the reproducibility of the data.

As observed using XTT assays, cell proliferation was not inhibited in *miR-221* and *miR-222* transfectants in comparison with mock- or miR-control-transfected cells (Figure 2A). However,

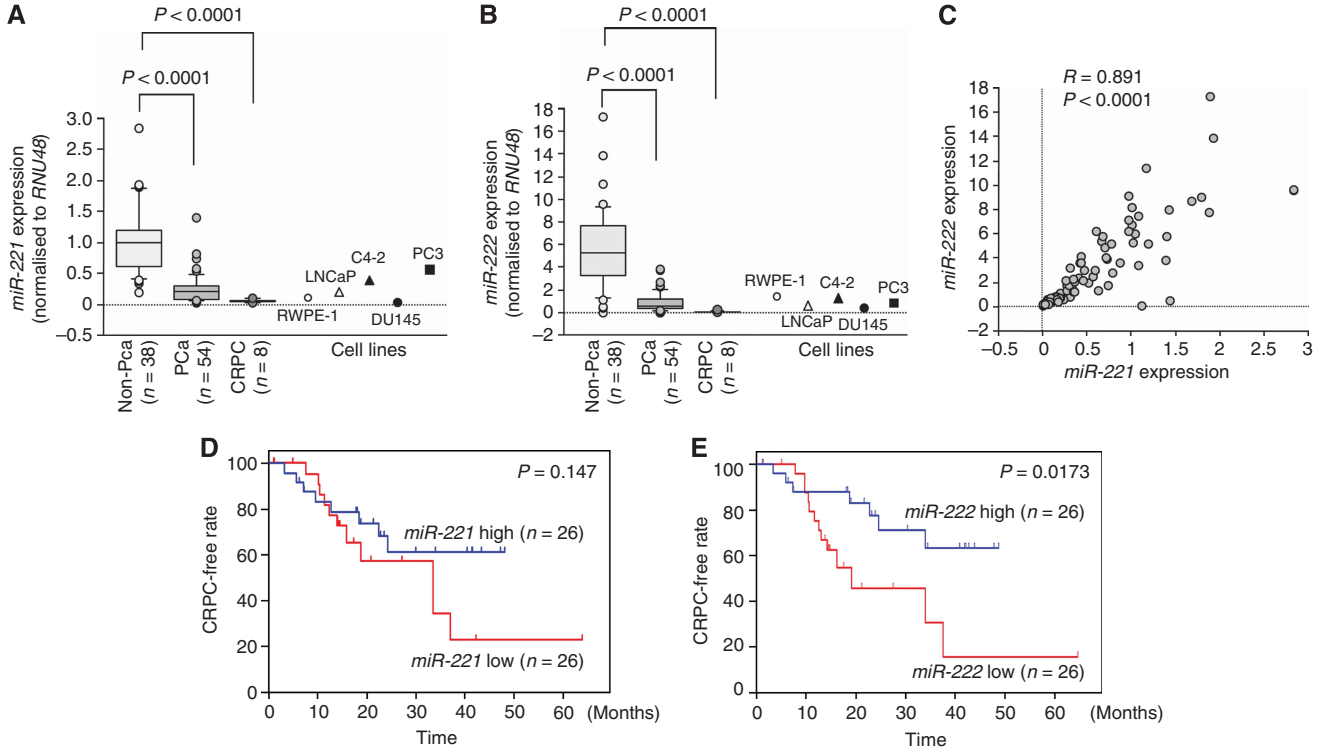

Figure 1. Expression levels of *miR-221/222* in prostate specimens and associations between *miR-221/222* expression and CRPC-free interval. Expression levels of *miR-221* (**A**) and *miR-222* (**B**) in clinical prostate specimens. *RNU48* was used for normalisation. (**C**) Correlations among the relative expression levels of *miR-221/miR-222*. Kaplan–Meier survival curves for CRPC progression-free survival based on (**D**) *miR-221* and (**E**) *miR-222* expression in PCa patients. *P*-values were calculated using the log-rank test.

Table 2. Univariate and multivariate Cox proportional analysis for the prediction of CRPC progression-free survival

	Univariate analysis			Multivariate analysis		
Covariant	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
miR-222	0.352	0.135–0.917	**0.0326**	0.206	0.067–0.637	**0.006**
cT stage	2.497	1.009–6.18	**0.0477**	1.311	0.4448–3.833	0.621
cN stage	3.768	0.873–16.269	0.0755	4.196	0.792–22.23	0.0918
cM stage	3.54	1.033–12.135	**0.0443**	2.618	0.607–11.299	0.1971
PSA at diagnosis	1	1–1.001	0.1674	1	1.000–1.001	0.3442
Gleason score	1.663	0.756–3.659	0.2061	1.433	0.637–3.223	0.3846
Age	1.014	0.951–1.081	0.6657	1.129	1.019–1.25	0.0198

Abbreviations: CI = confidence interval; CRPC = castration-resistant prostate cancer; HR = hazard ratio; PSA = prostate-specific antigen. $P<0.05$ is shown in bold characters.

miR-221 and *miR-222* transfection significantly inhibited cell migration as compared with mock- or miR-control-transfected cells ($P<0.0001$; Figure 2B). Similarly, Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in *miR-221* and *miR-222* transfectants in comparison with mock or miR-control transfectants ($P<0.0001$; Figure 2C).

In addition, we investigated cell cycle and apoptosis assay by using *miR-221* or *miR-222* transfectant in PC3 cells. No positive data were observed in both assays (Supplementary Figures 6 and 7).

Identification of target genes regulated by the *miR-221/222* cluster in PCa. To identify target genes of *miR-221/222*, we performed *in silico* analysis and oligomicroarray analysis. The TargetScan programme showed that 2275 genes had putative target sites for *miR-221/222* in their 3′ UTRs. To gain further insights into which genes were affected by the tumour-suppressive *miR-221/222* cluster in PCa, we investigated their expression status in PCa clinical specimens and examined gene expression profiles in the GEO database (accession numbers GSE29079) to evaluate upregulated genes in PCa specimens. Among the 2275 putative target genes of the *miR-221/222* cluster, 135 genes were significantly upregulated in PCa specimens compared with non-PCa tissues ($\log_2$ ratio >0.5). Finally, we performed genome-wide gene expression analysis using PC3 and DU145 cells (GEO accession number GSE56243). Genes downregulated ($\log_2$ ratio < -0.1) by transfection with *miR-221* and *miR-222* were selected as putative target genes. A total of 17 putative candidate genes for *miR-221/222* regulation were identified. In this study, we sorted these candidate genes in order of GEO expression data (Table 3), because we considered that high expression genes in cancer tissues functioned as an oncogene. As a result, *Ecm29* was the most upregulated gene in the putative candidate genes. Moreover, *Ecm29* has a conserved binding site for *miR-221/222*. We focused on *Ecm29* for further studies. Our strategy for selection of *miR-221/222* cluster-targeted genes is shown in Supplementary Figure 5.

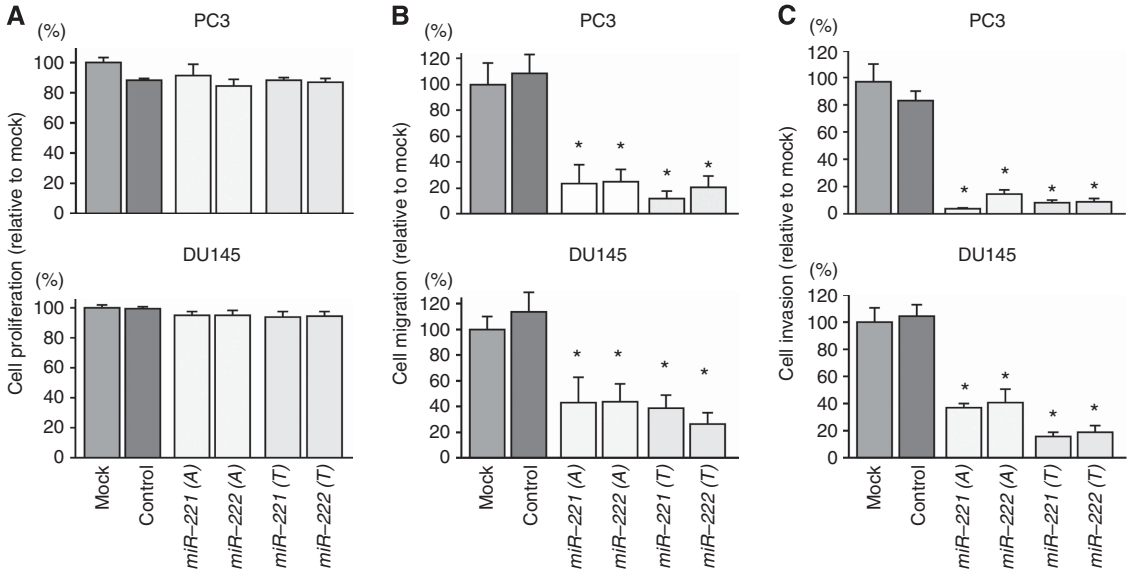

Figure 2. Effects of *miR-221/222* transfection on cell proliferation, migration, and invasion in PC3 and DU145 cells. (**A**) Cell proliferation was determined 72 h after transfection with *miR-221/222* using XTT assays. (**B**) Cell migration activity was determined 48 h after transfection with *miR-221/222* using migration assays. (**C**) Effects of *miR-221/222* transfection on cell invasion in PC3 and DU145 cells. Cell invasion activity was determined 48 h after transfection with *miR-221/222* using Matrigel invasion assays. *miR-221*-A and *miR-222*-A: Ambion, *miR-221*-T and *miR-222*-T: Thermo Scientific Dharmacon. *$P<0.0001$. Experiments were performed triplicate. The bars mean s.d.

***Ecm29* was a direct target of *miR-221/222* in PCa cells.** We performed real-time RT–qPCR and western blotting in PC3 and DU145 cells to investigate whether restoration of *miR-221* and *miR-222* altered the expression of the *Ecm29* gene and Ecm29 protein. The mRNA and protein expression levels of *Ecm29*/Ecm29 were significantly repressed in *miR-221/222* transfectants as compared with mock- or miR-control-transfected cells ($P<0.0001$; Figures 3A and B and Supplementary Figure 8).

Therefore, we next performed luciferase reporter assays in PC3 cells to determine whether *Ecm29* mRNA had target sites for *miR-221* and *miR-222*. The TargetScan database predicted that *miR-221* and *miR-222* bound at position 97–103 in the 3′ UTR of *Ecm29*. We used vectors encoding a partial wild-type sequence of the 3′ UTR of *Ecm29* mRNA, including the predicted *miR-221* and *miR-222* target site, or a vector lacking the *miR-221* and *miR-222* target site. We found that the luminescence intensity was significantly reduced by cotransfection with *miR-221* or *miR-222* and the vector carrying the wild-type 3′ UTR of *Ecm29*. On the other hand, the luminescence intensity was not decreased when the seed sequence of the target site was deleted from the vectors ($P<0.0001$; Figure 3C).

Effects of silencing *Ecm29* on cell proliferation, migration, and invasion in PCa cell lines. To investigate the functional role of *Ecm29*, we performed loss-of-function studies using si-*Ecm29* transfectants. First, we evaluated the knockdown efficiency of si-*Ecm29* transfection in PC3 and DU145 cells. RT–qPCR and western blotting indicated that si-*Ecm29* transfection effectively downregulated Ecm29 mRNA and protein expression in PC3 and DU145 cells (Figures 4A and B).

In functional assays, cell proliferation and migration were not inhibited by transfection with si-*Ecm29* in comparison with mock- or si-control-transfected DU145 cells (Figures 4C and D). However, cell invasion (Figure 4E) assays demonstrated that cancer cell invasion activity was significantly inhibited in si-*Ecm29* transfectants in comparison with mock- or si-control-transfected PC3 and DU145 cells.

Expression of *Ecm29* in clinical PCa specimens. A total of 20 PCa samples (Supplementary Table 1, Nos. 1–20), 20 non-PCa samples (Supplementary Table 1, Nos. 55–74), and 8 CRPC samples were used to analyse *Ecm29* mRNA expression in this study. RT-qPCR analysis showed that the expression of *Ecm29* mRNA was significantly higher in clinical PCa specimens than in non-PCa specimens ($P=0.0101$; Figure 5A). Furthermore, expression of *Ecm29* mRNA was significantly higher in CRPC specimens than in PCa and non-PCa specimens ($P=0.0125$ and $P<0.0001$, respectively; Figure 5A). In these 48 clinical specimens, Spearman's rank test showed that there was a negative correlation between the expression level of *miR-221* and that of *Ecm29* ($R=-0.548$ and $P=0.0002$; Figure 5C). Similarly, a negative correlation was found between the expression level of *miR-222* and that of *Ecm29* ($R=-0.484$ and $P=0.0009$; Figure 5D). Immunohistochemical staining of Ecm29 in CRPC specimens demonstrated high expression of Ecm29 in the cytoplasm of CRPC cells and low expression in the nucleus (Figure 5B).

DISCUSSION

A growing body of evidence has shown that miRNAs are involved in several biological processes and are tightly correlated with human oncogenesis and metastasis (Nelson and Weiss, 2008). Recent studies from our laboratory have identified a variety of novel molecular targets and pathways regulated by tumour-suppressive miRNAs in PCa based on PCa miRNA signatures (Goto *et al*, 2014b; Nishikawa *et al*, 2014). Moreover, we have begun to analyse clustered miRNAs and have reported that several miRNA clusters, including *miR-1/133*, *miR-23b/27b/24-1*, and *miR-143/145*, act as tumour suppressors and contribute substantially to PCa oncogenesis and metastasis (Kojima *et al*, 2012, 2014; Goto *et al*, 2014a). Although elucidation of the molecular networks in CRPC specimens is needed to improve therapies for and prevention of the disease, advancement of genomic analysis is difficult because of the challenges with obtaining clinical CRPC specimens.

Evaluation of miRNA expression signatures using CRPC specimens is an indispensable tool for cancer research. In this study, we have constructed a new CRPC miRNA signature and

Table 3. Downregulated genes in *miR-221/222* transfectants and upregulated genes in the GEO database

Entrez gene ID	Symbol	Gene name	Location	PC3 *miR-221* transfectant	PC3 *miR-222* transfectant	DU145 *miR-221* transfectant	DU145 *miR-222* transfectant	Average	GEO fold change	No. of conserved sites	No. of poorly conserved sites
23392	KIAA0368	Ecm29 (KIAA0368)	9q31.3	−1.28	−1.05	−0.82	−0.62	−0.94	0.999	1	0
159195	USP54	Ubiquitin-specific peptidase 54	10q22.2	−0.73	−0.65	−0.19	−0.15	−0.43	0.869	0	1
51809	GALNT7	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 7 (GalNAc-T7)	4q34.1	−0.75	−0.67	−0.85	−0.94	−0.80	0.833	0	1
10238	DCAF7	DDB1- and CUL4-associated factor 7	17q23.3	−0.78	−0.87	−0.58	−0.47	−0.67	0.767	1	1
55920	RCC2	Regulator of chromosome condensation 2	1p36.13	−0.48	−0.53	−0.12	−0.18	−0.33	0.734	0	1
23097	CDK19	Cyclin-dependent kinase 19	6q21	−2.77	−2.18	−0.56	−0.18	−1.42	0.732	1	2
58508	MLL3	Myeloid/lymphoid or mixed-lineage leukaemia 3	7q36.1	−1.44	−1.40	−0.42	−0.28	−0.88	0.697	0	1
9870	KIAA0317	KIAA0317	14q24.3	−0.67	−0.47	−0.36	−0.41	−0.48	0.621	0	1
54874	FNBP1L	Formin-binding protein 1-like	1p22.1	−1.20	−1.25	−0.22	−0.21	−0.72	0.613	0	1
55884	WSB2	WD repeat and SOCS box containing 2	12q24.23	−2.64	−2.71	−1.58	−1.21	−2.03	0.569	1	1
6319	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	10q24.31	−1.78	−1.81	−0.49	−0.15	−1.06	0.545	0	1
2017	CTTN	Cortactin	11q13.3	−1.12	−1.01	−0.32	−0.14	−0.65	0.542	0	1
5451	POU2F1	POU class 2 homeobox 1	1q24.2	−0.46	−0.43	−0.21	−0.26	−0.34	0.524	0	1
204851	HIPK1	Homeodomain interacting protein kinase 1	1p13.2	−2.00	−1.70	−0.81	−0.56	−1.27	0.519	1	0
9265	CYTH3	Cytohesin 3	7p22.1	−0.53	−0.43	−0.58	−0.39	−0.48	0.518	0	1
23126	POGZ	Pogo transposable element with ZNF domain	1q21.3	−0.64	−0.80	−0.26	−0.16	−0.46	0.515	1	0
2799	GNS	Glucosamine (*N*-acetyl)-6-sulfatase	12q14.3	−0.65	−1.16	−0.16	−0.29	−0.57	0.513	0	1

identified several downregulated miRNAs in CRPC tissues compared with PCa and normal prostate tissues. According to our previous PCa signatures and other studies, the clustered miRNAs *miR-221* and *miR-222* were significantly downregulated in PCa specimens (Porkka *et al*, 2007; Ambs *et al*, 2008; Schaefer *et al*, 2010; Szczyrba *et al*, 2010; Carlsson *et al*, 2011; Fuse *et al*, 2012; Wach *et al*, 2012). Furthermore, our present CRPC signature revealed that both *miR-221* and *miR-222* were also significantly reduced in CRPC specimens. These findings suggested that downregulation of the molecular networks regulated by the *miR-221/222* cluster contributes substantially to PCa and CRPC progression.

Currently, a variety of diverse treatment options are available for patients with PCa, particularly those with localised PCa (Heidenreich *et al*, 2014b). However, it is not clear which treatment is suitable for achieving an optimal prognosis in patients with high-risk PCa. Prediction of the time to progression to CRPC under ADT using the expression status of miRNAs from needle biopsies during initial diagnosis will improve the accuracy of treatment decisions for patients with high-risk PCa. We found that *miR-222* expression status was a good prognostic marker for time to progression to CRPC using hormone-naïve prostate biopsy specimens. A recent study demonstrated that *miR-221* expression is progressively reduced in aggressive PCa and predicts recurrence-free survival after radical prostatectomy in patients with high-risk PCa (Kneitz *et al*, 2014). These results strongly suggest that the *miR-221/222* cluster may be useful in PCa diagnosis. A large-scale cohort study will be necessary to determine whether *miR-221* and *miR-222* are effective markers for CRPC-free interval.

In this study, we also investigated the functional significance of *miR-221* and *miR-222* using two different types of *miR-221* and *miR-222* mimic in gain-of-function studies. We found that restoration of *miR-221* and *miR-222* using these four constructs significantly inhibited cancer cell migration and invasion. These results strongly suggested that *miR-221* and *miR-222* functioned as tumour suppressors in PCa cells. Our expression data of *miR-221/222* transfectants in PC3 cells (GEO accession number GSE56243) demonstrated that several downregulated genes were categorised to 'ECM-receptor interaction', 'Cell adhesion molecules', and 'focal adhesion pathways'. These differentially expressed genes were deeply contributed to cancer cell migration and invasion in PCa. Moreover, a recent study showed that *miR-221* regulates cell growth, invasion, and apoptosis by targeting the oncogenes *SOCS3* and *IRF2*, suggesting that *miR-221* has a tumour-suppressive role in PCa cells (Kneitz *et al*, 2014). The tumour-suppressive functions of the *miR-221/222* cluster have been reported in other types of cancers, such as tongue squamous cell carcinomas and gastric cancers (Garofalo *et al*, 2012). These findings strongly support that the *miR-221/222* cluster functions as a tumour suppressor in PCa cells.

In contrast to tumour-suppressive function of *miR-221/222*, previous studies have shown that *miR-221* and *miR-222* act as oncogenes. Upregulation of *miR-221* and *miR-222* were observed in CRPC cell lines and these miRNAs promote cancer cell proliferation (Sun *et al*, 2014). Furthermore, *in vivo* mouse model study demonstrated that inhibition of *miR-221/222* in PC3 cells with LNA antisense oligonucleotides reduced tumour growth (Mercatelli *et al*, 2008). Several studies of breast cancer have demonstrated that the *miR-221/222* cluster acts as an onco-miR, targeting tumour-suppressive genes, such as $p27^{Kip1}$ and oestrogen receptor alpha (Shah and Calin, 2011). The oncogenic functions of *miR-221* and *miR-222* have also been reported in glioblastoma and melanoma (Garofalo *et al*, 2012). It is unclear why the expression statuses of these miRNAs vary in different types of cancer cells. Detailed molecular studies of the expression control of these miRNAs by the cancer types is necessary.

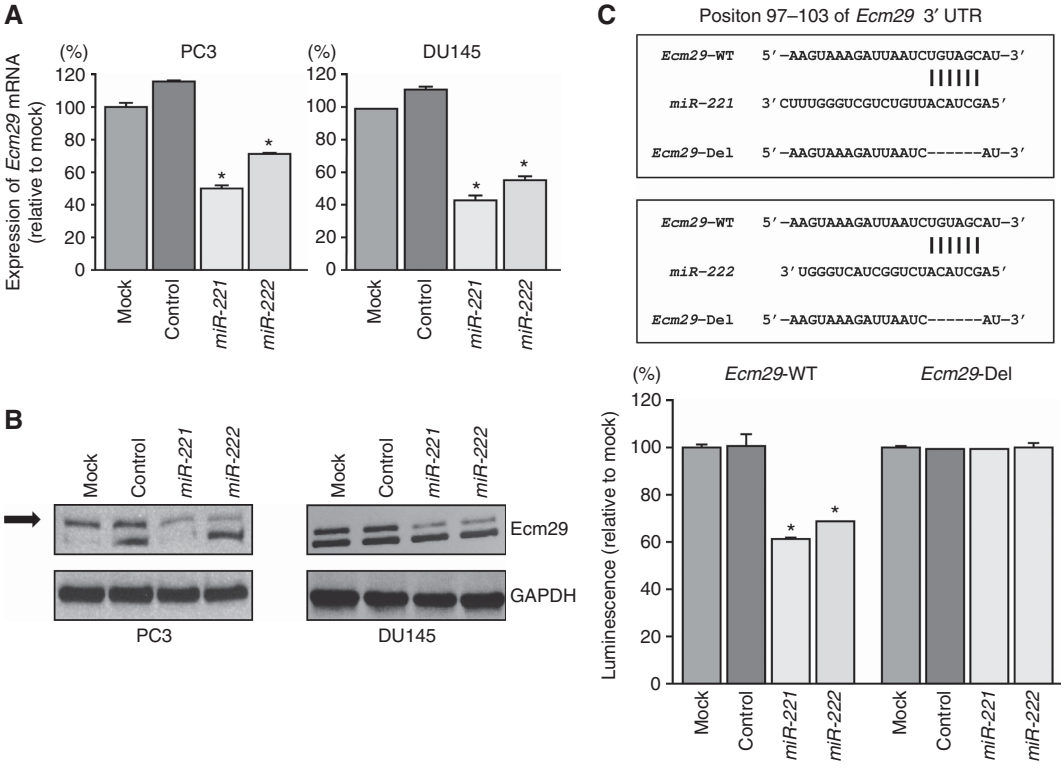

Figure 3. Downregulation of *Ecm29* expression by *miR-221/222* in PC3 and DU145 cells. (**A**) *Ecm29* mRNA expression 72 h after transfection with *miR-221/222*. GUSB was used as an internal control. *$P<0.0001$. (**B**) Ecm29 protein expression 48 h after transfection with *miR-221/222*. GAPDH was used as a loading control. (**C**) *miR-221/222*-binding sites in *Ecm29* mRNA. Luciferase reporter assays were carried out using a vector encoding the putative *miR-221/222* target site in the *Ecm29* 3′-UTR (position 97–103) for wild-type and deletion constructs. *$P<0.0001$. Experiments were performed triplicate. The bars mean s.d.

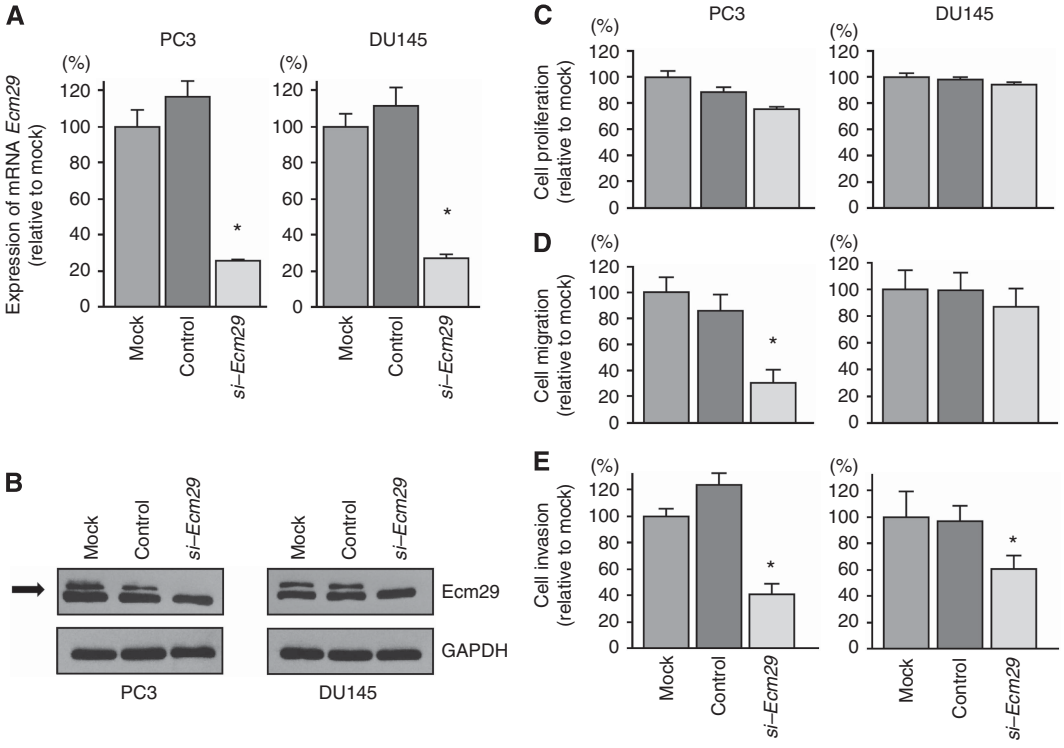

Figure 4. Effects of silencing *Ecm29* mRNA and protein expression by *si-Ecm29* transfection in PCa cells on cell proliferation, migration, and invasion in PCa cell lines. (**A**) *Ecm29* mRNA expression was determined at 72 h after transfection with *si-Ecm29*. *GUSB* was used as an internal control. (**B**) Ecm29 protein expression was evaluated by western blotting at 48 h after transfection with *si-Ecm29*. GAPDH was used as a loading control. *$P<0.0001$. (**C**) Cell proliferation was determined by XTT assays. (**D**) Cell migration activity was determined by wound healing assays. (**E**) Cell invasion activity was determined by Matrigel invasion assays. *$P<0.0001$. mock: untransfected cells, control: control siRNA-transfected cells. Experiments were performed triplicate. The bars mean s.d.

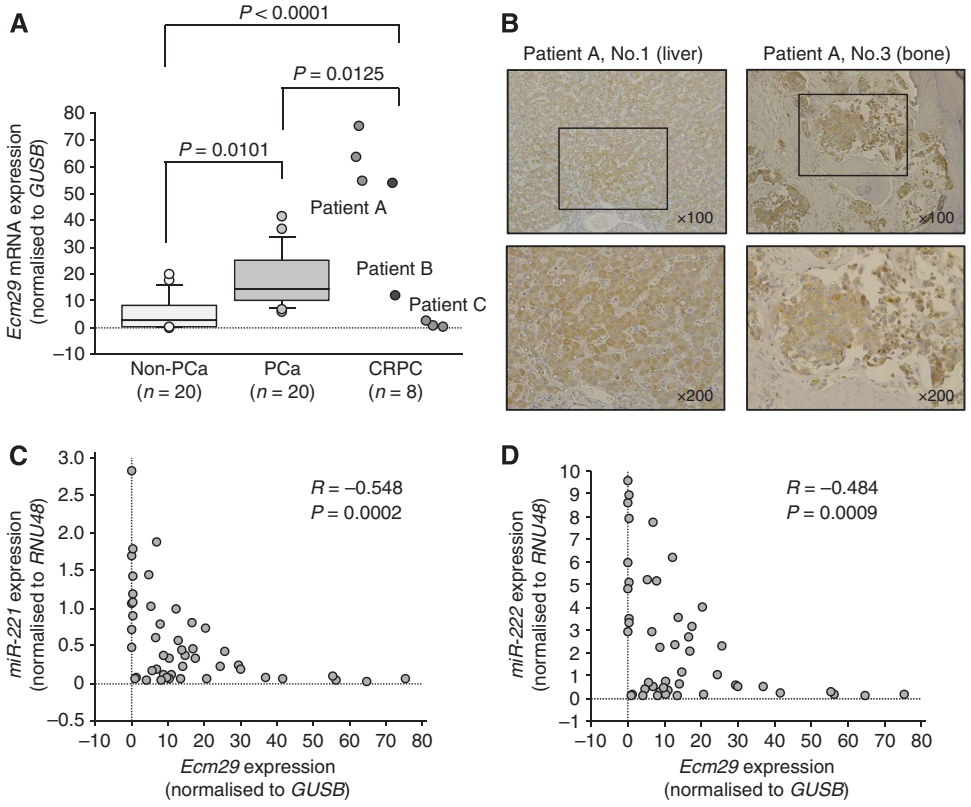

Figure 5. Expression of *Ecm29* in clinical PCa specimens. (**A**) *Ecm29* mRNA expression levels were determined by qRT–PCR analysis in PCa, non-PCa, and CRPC specimens. *GUSB* was used as an internal control. (**B**) Ecm29 expression in CRPC specimens. High expression of Ecm29 was found in the cytoplasm of CRPC cells, whereas the expression was low in the nucleus. (**C**) Inverse correlation between *Ecm29* mRNA and *miR-221* expression. (**D**) Inverse correlation between *Ecm29* mRNA and *miR-222* expression.

Regulation mechanisms of *miR-221/222* cluster are reported in some articles. As for colorectal cancer, *miR-221/222* is upregulated in cancer tissue and positively regulates NF-kB-STAT3 pathway and vice versa, forming feedback loop, which has critical role for colorectal cancer growth (Di Leva *et al*, 2010). Similarly, in breast cancer, *miR-221/222* directly regulates ERα and ERα represses *miR-221/222* expression recruiting co-repressor proteins (Liu *et al*, 2014). The mechanisms of downregulation of *miR-221/222* in PCa are yet to be clear. Thus, elucidation of the epigenetic mechanisms controlling the expression of clustered miRNAs in different types of cancer cells is an important theme in cancer research.

Normal regulatory mechanisms can be disrupted by the aberrant expression of tumour-suppressive or oncogenic miRNAs in cancer cells. Therefore, one of the next problems to address is identification of oncogenic genes that are regulated by the *miR-221/222* cluster in PCa cells. We selected 17 putative candidate genes using a combination of *in silico* and genome-wide gene expression analyses in the present study. Among them, we focused on the *Ecm29* gene as a responsible oncogene in PCa oncogenesis and metastasis because the functional significance of *Ecm29* is not well understood in PCa cells. Ecm29 is a conserved 210-kDa protein composed almost entirely of HEAT-like repeats (Gorbea *et al*, 2004). It is a scaffold protein that links the 26S proteasome to motor proteins, resulting in inhibition of proteasome activity and functioning as a proteasome quality control protein (Gorbea *et al*, 2010; Lehmann *et al*, 2010; De La Mota-Peynado *et al*, 2013). However, the precise mechanisms of *Ecm29* in cancer cells remain unknown. Our data demonstrated that *Ecm29* is significantly upregulated in PCa clinical specimens. Also, overexpressed *Ecm29* was observed in some CRPC specimens. Large number of PCa and CRPC specimens are necessary to investigate the clinical significance of *Ecm29* expression. Gene expression database search showed that upregulation of *Ecm29* was observed in other types of cancer, such as muscle invasive bladder cancer and pancreatic cancer (GSE15471 and GSE3167). These expression results support our present data of PCa (Supplementary Figure 9). Knockdown of *Ecm29* by siRNAs significantly inhibited migration of PC3 cells (not DU145 cells) and inhibited invasion of both PC3 and DU145 cells. Our data are the first report demonstrating that *Ecm29* contributed to cancer cell migration and invasion in PCa cell. We showed that *Ecm29* was under direct regulation of *miR-221/222* in PCa cells, however, many targets of *miR-221/222* regulation are still unclear. Elucidation of other candidate targets of the tumour-suppressive *miR-221/222* cluster regulation presented in this study may improve our understanding of PCa progression and metastasis.

CONCLUSIONS

Downregulation of the *miR-221/222* cluster was identified based on the miRNA expression signature of CRPC in this study. Moreover, Kaplan–Meier survival curves showed that low expression of *miR-222* predicted a short duration of progression to CRPC. *miR-221* and *miR-222* were shown to function as tumour suppressors in PCa cells. To the best of our knowledge, this is the first report demonstrating that the tumour-suppressive *miR-221/222* cluster directly regulated *Ecm29* in PCa cells. The identification of novel molecular pathways and targets regulated by the *miR-221/222* cluster based on CRPC signatures may lead to a better

understanding of PCa and the development of new therapeutic strategies to treat this disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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