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ORIGINAL ARTICLE

Tumor suppressive microRNAs (*miR-222* and *miR-31*) regulate molecular pathways based on microRNA expression signature in prostate cancer

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microRNAs (miRNAs) have key roles in human tumorigenesis, tumor progression and metastasis. miRNAs are aberrantly expressed in many human cancers and can function as tumor suppressors or oncogenes that target many cancer-related genes. This study seeks to identify novel miRNA-regulated molecular pathways in prostate cancer (PCa). The miRNA expression signature in clinical specimens of PCa showed that 56 miRNAs were significantly downregulated in PCa compared with non-PCa tissues. We focused on the top four downregulated miRNAs (*miR-187*, *miR-205*, *miR-222* and *miR-31*) to investigate their functional significance in PCa cells. Expression levels of these four miRNAs were validated in PCa specimens (15 PCa tissues and 17 non-PCa tissues) to confirm that they were significantly reduced in these PCa tissues. Gain-of-function analysis demonstrated that *miR-222* and *miR-31* inhibited cell proliferation, invasion and migration in PCa cell lines (PC3 and DU145), suggesting that *miR-222* and *miR-31* may act as tumor suppressors in PCa. Genome-wide gene expression analysis using *miR-222* or *miR-31* transfectants to identify the pathways they affect showed that many cancer-related genes are regulated by these miRNAs in PC3 cells. Identification and categorization of the molecular pathways regulated by tumor suppressive miRNAs could provide new information about the molecular mechanisms of PCa tumorigenesis.
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Keywords: microRNA; *miR-222*; *miR-31*; prostate cancer; tumor suppressor

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer and second leading cause of cancer death among men in developed countries.[1] In early-stage PCa, more than 90% of patients initially respond to the therapeutic use of androgen deprivation; however, many cases become refractory and progress to androgen-independent PCa.[2] Hormone-refractory PCa often involves disease progression and metastasis to the bones or other sites and is currently difficult to treat. As most clinical trials for advanced PCa have shown limited benefits,[3,4] new prognostic markers and effective treatment strategies are urgently needed. Understanding the molecular pathways underlying PCa could significantly improve diagnosis, therapy and disease prevention.

miRNAs are a class of small non-coding RNA molecules consisting of 19–22 nucleotides that are involved in a variety of biological processes, including development, differentiation, apoptosis and cell proliferation. miRNAs regulate gene expression through translational repression and mRNA cleavage[5] with bioinformatic predictions indicating that miRNAs regulate more than 60% of protein coding genes.[6] To date, 1527 human miRNAs have been registered in the miRBase release 18.0 (http://microrna.sanger.ac.uk/). miRNAs also contribute to the initiation and development of various types of cancers. Many human cancers have aberrant expression of miRNAs, which can function either as tumor suppressors or oncogenes.[7,8] In cancer pathways, normal regulatory mechanisms are disrupted by altered expression of tumor suppressive or oncogenic miRNAs.

Recently, we sequentially identified tumor suppressive miRNAs in several types of cancers, such as head and neck squamous cell carcinoma,[9,10] esophageal squamous cell carcinoma,[11] bladder cancer,[12,13] renal cell carcinoma[14] and PCa[15] and described the molecular pathways they regulate. Our analysis of miRNA-regulated cancer pathways provided novel insights for diagnostic and therapeutic strategies for these cancers.

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In this study we identified tumor suppressive miRNAs in PCa based on the miRNA expression signature in clinical PCa specimens. The evaluation of 778 mature miRNAs from PCa specimens revealed that 57 miRNAs had significantly reduced expression. Gain-of-function analysis demonstrated that *miR-222* and *miR-31* inhibited cell proliferation, invasion and migration in PCa cell lines (PC3 and DU145). Furthermore, we used genome-wide gene expression analysis to identify target genes of tumor suppressive *miR-222* or *miR-31* and determine whether these miRNAs regulate cancer pathways. The identification of tumor suppressive miRNAs and their corresponding molecular pathways could provide new insights into PCa tumorigenesis.

MATERIALS AND METHODS

Clinical prostate specimens

Clinical specimens were obtained from patients at Teikyo University Chiba Medical Center Hospital from 2008 to 2010. All patients had elevated serum levels of prostate-specific antigen and had undergone transrectal prostate needle biopsy. Prostatic cancerous tissues (PCa, $n=20$) and non-cancerous tissues (non-PCa, $n=22$) were used in this study. Among these, five PCa (no. 1–5) and five non-PCa (no. 21–25) samples were used for miRNA expression analysis and the remainder was used to validate selected miRNA expression levels. Patient characteristics and representative pathological features are shown in Table 1 and Figure 1, respectively. Written consent for tissue donation for research purposes was obtained from patients before tissue collection. The protocol was approved by the Institutional Review Board of Teikyo University. To confirm tissue composition, a pair of needle biopsy specimens was collected from the same region in the patients, and one sample was subjected to pathological validation. No cancerous tissue was found in non-PCa specimens.

miRNA expression signatures and data normalization

miRNA expression patterns were evaluated using the TaqMan LDA Human microRNA Panel v2.0 with a total of 778 miRNAs investigated in the screen (Applied Biosystems, Foster City, CA, USA). The assay consisted of two steps: cDNA generation by reverse transcription and a TaqMan real-time PCR assay. The description of real-time PCR and the list of human miRNAs can be found on the company's website (http://www.appliedbiosystems.com). An 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 <0.05 was used to narrow the candidates after normalization of the raw data. Normalization was done with respect to *RNU6B*.

Cell culture

We used human PCa cell lines (PC3 and DU145), which were obtained from the American Type Culture Collection (Manassas, VA, USA) and 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.

RNA extraction

Total RNA including miRNA was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA integrity was checked with the RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer (Agilent Technologies).

Quantitative real-time RT–PCR

For miRNA quantitation we used TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems). The cDNA strand was synthesized from total RNA (10 ng). Thermal cycling conditions were as follows: 95 °C for 15 s and 63 °C for 1 min. The expression levels of *miR-187* (assay ID: 001193), *miR-205* (assay ID: 000509), *miR-222* (assay ID: 002276) and *miR-31* (assay ID: 002279) were normalized with respect to *RNU6B* (assay ID: 001973). All reactions were performed in triplicate and negative controls lacking cDNA were included. The data were analyzed with the delta-delta Ct method to calculate the fold-change. For the internal controls, we examined the unevenness between the clinical samples and adapted them for use with *RNU6B* in this study.

Table 1 Patients' characteristics

						TNM classification		
No.	*PCa or non-PCa*	*Age*	*PSA*	*Gleason score*	*Stage*	*T*	*N*	*M*
1	PCa	78	819	4+4	D2	4	1	1
2	PCa	70	1100	4+5	D2	4	0	1
3	PCa	62	409	4+4	D2	4	0	1
4	PCa	75	1200	4+5	D2	4	1	1
5	PCa	59	315	4+4	D2	4	0	1
6	PCa	77	17.8	4+5	D1	3a	1	0
7	PCa	67	244	4+4	D2	4	1	1
8	PCa	70	395	4+4	D2	3	1	1
9	PCa	83	49.9	4+5	C	3	0	0
10	PCa	68	212	4+4	D1	3b	1	0
11	PCa	80	589	4+5	D1	3b	1	0
12	PCa	72	2530	4+5	D2	3	1	1
13	PCa	76	12.5	4+5	D2	3b	1	1
14	PCa	67	153	4+4	D2	4	1	1
15	PCa	82	808.8	4+5	D2	4	1	1
16	PCa	88	50.5	4+4	C	3a	0	0
17	PCa	69	3.45	4+3	C	3a	0	0
18	PCa	64	486	4+5	D2	4	1	1
19	PCa	74	60.8	5+5	D2	4	1	1
20	PCa	63	49.6	4+4	D1	3b	1	0
21	non-PCa	67	7.63	—	—	—	—	—
22	non-PCa	61	4.39	—	—	—	—	—
23	non-PCa	66	5.76	—	—	—	—	—
24	non-PCa	59	6.34	—	—	—	—	—
25	non-PCa	71	11.4	—	—	—	—	—
26	non-PCa	85	10.1	—	—	—	—	—
27	non-PCa	61	2.88	—	—	—	—	—
28	non-PCa	55	11.2	—	—	—	—	—
29	non-PCa	75	9	—	—	—	—	—
30	non-PCa	58	5.87	—	—	—	—	—
31	non-PCa	67	22	—	—	—	—	—
32	non-PCa	63	14.2	—	—	—	—	—
33	non-PCa	66	7.33	—	—	—	—	—
34	non-PCa	69	7.92	—	—	—	—	—
35	non-PCa	68	7.24	—	—	—	—	—
36	non-PCa	53	4.33	—	—	—	—	—
37	non-PCa	80	9.43	—	—	—	—	—
38	non-PCa	62	5.14	—	—	—	—	—
39	non-PCa	63	10	—	—	—	—	—
40	non-PCa	62	5.11	—	—	—	—	—
41	non-PCa	74	8.3	—	—	—	—	—
42	non-PCa	60	13.5	—	—	—	—	—

Abbreviations: non-PCa, non-prostate cancer; PCa, prostate cancer; PSA, prostate-specific antigen.

Mature miRNA transfection

Pre-miR and negative-control miRNA (Applied Biosystems) were used for gain-of-function experiments. As previously described,[13] PC3 and DU145 cells were transiently transfected with precursors of *miRNA-187*, *miR-205, miR-222, miR-31* or negative control using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Mock transfections, which had only the transfection

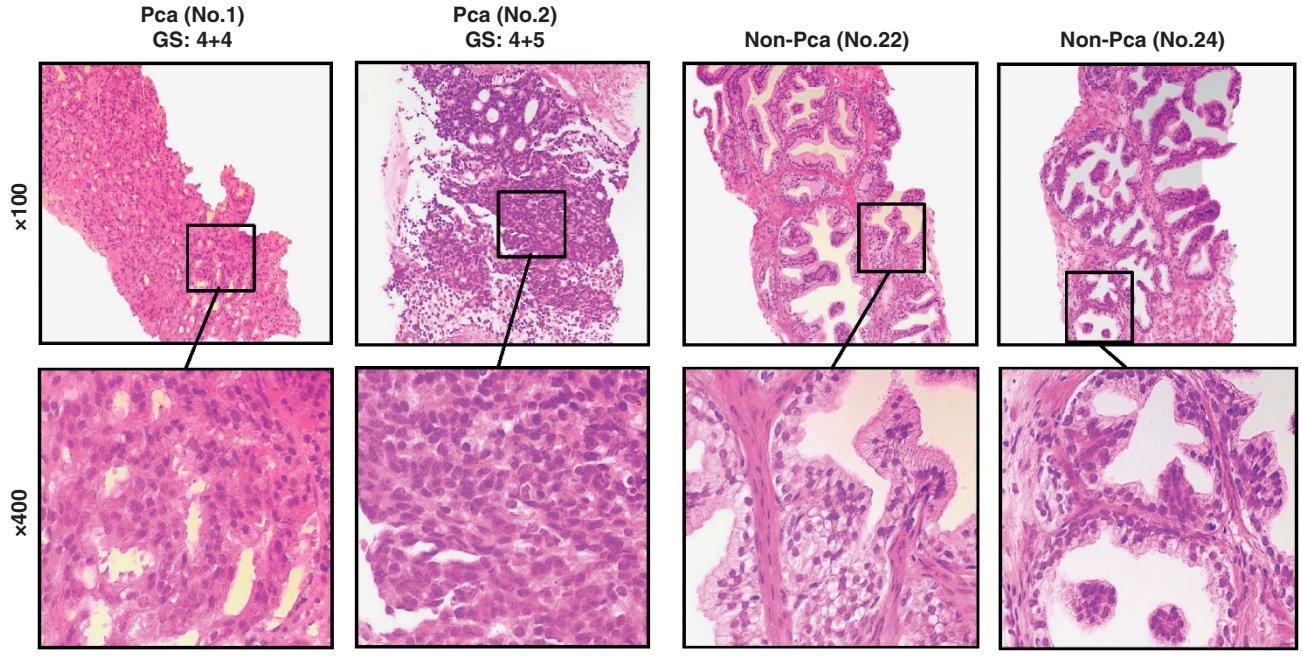

Figure 1 HE staining of the prostate needle biopsy cores showed massive inbiltration of high grade PCa cells (no. 1 and 2) and normal prostate tissues (no. 22 and 24).

reagent, were also used as controls. The transfection efficiency of miRNA into cancer cells was evaluated by downregulation of *PTK9* mRNA by *miR-1* transfection level as described previously.[13] Cells were seeded in six-well plates for mRNA and wound healing assays (25×10^4 cells per well) and in 96-well plates for XTT assays (3000 cells per well).

Cell proliferation, migration and invasion assays

Cell proliferation was determined using an XTT assay (Roche Applied Sciences, Tokyo, Japan) performed according to the manufacturer's instructions. Cell migration activity was evaluated with a wound-healing assay. Cells were plated in six-well dishes, and the cell monolayers were scraped using a P-20 micropipette tip. The initial gap length (0 h) and residual gap length 24 h after wounding were calculated from photomicrographs as described previously.[15,16] A cell invasion assay was carried out using modified Boyden Chambers consisting of transwell-precoated Matrigel membrane filter inserts with 8 μm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). Minimum essential medium containing 10% fetal bovine serum in the lower chamber served as the chemoattractant, as described previously.[15,16] All experiments were performed in triplicate.

Statistical analysis

The relationship between two variables and the numerical values obtained by real-time RT–PCR were analyzed using the Mann–Whitney *U*-test. The relationship among three variables and the numerical values were analyzed using the Bonferroni-adjusted Mann–Whitney *U*-test. Expert StatView analysis software (version 4; SAS Institute Inc., Cary, NC, USA) was used in both cases. For comparisons among three variables, a nonadjusted statistical level of significance of $P<0.05$ corresponds to a Bonferroni-adjusted level of $P<0.0167$.

Identification of miR-222 or miR-31 regulated target genes and bioinformatic analysis

To gain further insight into which genes were affected by *miR-222 or miR-31*, we performed genome-wide gene expression analysis using *miR-222* or *miR-31* transfectants in PC cells. Oligo-microarray human 44 K (Agilent) was used for gene expression analysis, and procedure of the experiment as described previously.[15,16]

To identify the biological processes or pathways potentially regulated by selected miRNAs, we performed GeneCodis analysis (http://genecodis.dacya.ucm.es/) with our predicted target list. Then, to develop the networks among the miRNAs and their target genes, we analyzed and characterized those genes in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway categories.

RESULTS

Identification of downregulated miRNAs in PCa in miRNA signatures

We evaluated mature miRNA expression levels of prostate clinical specimens (five PCa tissues, no. 1–5, and five non-PCa tissues, no. 21–25) by miRNA signatures (Table 1). Figure 1 shows the typical needle biopsies that used for expression analysis in this study. Expression signatures revealed that 56 miRNAs were downregulated in PCa samples (Table 2). Of those, the top four miRNAs (*miR-187*, *miR-205*, *miR-222* and *miR-31*) were subjected to further study. The fifth most downregulated miRNA, *miR-133a*, was excluded as it was detected as a tumor suppressive miRNA in PCa in our previous study.[15]

Validation of *miR-187*, *miR-205*, *miR-222* and *miR-31* expression levels by quantitative stem-loop RT–PCR

Relative expression levels of the top four downregulated miRNAs (*miR-187*, *miR-205*, *miR-222* and *miR-31*) were analyzed in 15 PCa clinical samples compared with 17 non-PCa clinical samples (Table 1). All of these miRNAs were significantly downregulated in the PCa specimens (Figure 2). In the PCa cell line PC3, the expression levels of these four miRNAs were low and nearly equivalent to those of the PCa clinical samples (data not shown).

Effects of *miR-187*, *miR-205*, *miR-222* and *miR-31* restoration on cell proliferation, migration and invasion in PCa cell lines

To investigate the functional role of the four downregulated miRNAs in PCa, we performed gain-of-function studies using mature miRNA

Table 2 Downregulated miRNAs in PCa

miRNA	*Log2 ratio (PCa/non-PCa)*	P-*value*	*non-PCa*	*PCa*
hsa-miR-187	−4.78	0.008	1.835	0.067
hsa-miR-205	−4.72	0.007	13.311	0.504
hsa-miR-222	−3.88	0.005	547.451	37.090
hsa-miR-31	−3.55	0.005	24.270	2.075
hsa-miR-133a	−3.50	0.031	65.008	5.739
hsa-miR-224	−3.17	0.013	1.464	0.163
hsa-miR-196b	−2.95	0.033	10.595	1.367
hsa-miR-455-5p	−2.94	0.006	1.894	0.246
hsa-miR-30a*	−2.71	0.020	39.956	6.107
hsa-miR-139-5p	−2.70	0.008	15.533	2.385
hsa-miR-221	−2.67	0.023	8.681	1.364
hsa-miR-30e*	−2.64	0.013	28.765	4.606
hsa-miR-574-3p	−2.48	0.038	128.315	22.962
hsa-miR-133b	−2.48	0.001	1.547	0.277
hsa-miR-409-3p	−2.30	0.015	4.044	0.822
hsa-miR-411	−2.29	0.007	1.080	0.221
hsa-miR-320	−2.24	0.022	89.062	18.840
hsa-miR-125a-5p	−2.23	0.036	9.848	2.096
hsa-miR-28-3p	−2.23	0.004	31.722	6.773
hsa-miR-1	−2.20	0.003	1.217	0.264
hsa-miR-23b	−2.18	0.012	4.618	1.020
hsa-miR-376c	−2.17	0.026	2.332	0.520
hsa-miR-135a	−2.15	0.013	2.182	0.491
hsa-miR-193b	−2.09	0.024	49.792	11.655
hsa-miR-146b-5p	−2.02	0.002	24.730	6.109
hsa-miR-132	−1.99	0.001	13.737	3.461
hsa-miR-218	−1.96	0.009	6.476	1.665
hsa-miR-16	−1.95	0.011	279.520	72.122
hsa-miR-152	−1.94	0.005	7.857	2.046
hsa-miR-146a	−1.91	0.007	36.191	9.612
hsa-miR-145	−1.85	0.010	373.233	103.866
hsa-miR-135a*	−1.83	0.001	4.457	1.249
hsa-miR-223	−1.82	0.010	82.193	23.350
hsa-miR-200b*	−1.81	0.047	1.730	0.494
hsa-miR-24	−1.73	0.007	670.472	201.765
hsa-miR-324	−1.63	0.004	2.708	0.873
hsa-miR-378	−1.63	0.009	13.683	4.418
hsa-miR-99b	−1.57	0.009	9.095	3.053
hsa-miR-766	−1.57	0.003	1.458	0.490
hsa-let-7c	−1.57	0.008	13.069	4.396
hsa-let-7b	−1.56	0.010	37.451	12.682
hsa-miR-26a	−1.51	0.019	75.647	26.570
hsa-miR-29a	−1.50	0.024	159.548	56.285
hsa-miR-193a-5p	−1.49	0.020	2.439	0.866
hsa-miR-34a*	−1.49	0.013	1.261	0.450
hsa-miR-766	−1.44	0.007	1.339	0.494
hsa-miR-30c	−1.40	0.010	120.983	45.978
hsa-miR-27b	−1.38	0.048	5.626	2.165
hsa-miR-374b	−1.37	0.026	23.064	8.904
hsa-miR-99a*	−1.37	0.024	3.916	1.518
hsa-let-7e	−1.30	0.036	20.608	8.344
hsa-miR-532-3p	−1.28	0.019	4.803	1.979
hsa-miR-197	−1.25	0.028	3.035	1.274
hsa-miR-768-3p	−1.24	0.013	33.748	14.261
hsa-miR-331-3p	−1.24	0.033	19.195	8.111
hsa-miR-203	−1.21	0.049	6.184	2.674

Abbreviations: miR, micro RNA; non-PCa, non-prostate cancer; PCa, prostate cancer.
PCa: average of the expression levels of PCa tissues (patient number 1–5). non-PCa: average of the expression levels of non-PCa tissues (patient number 21–25).

transfection in the PCa cell lines, PC3 and DU145. An XTT assay showed significant inhibition of cell proliferation in *miR-222*- or *miR-31*-transfectants in both PC3 and DU145 cells compared with mock transfectants (percentage of cell viability relative to mock transfectants was 82.7 ± 1.3% for *miR-222* transfectants in PC3, and 63.6 ± 2.3% for *miR-31* transfectants in PC3; 81.1 ± 4.2% for *miR-222* transfectants in DU145, and 86.3 ± 3.4% for *miR-31* transfectants in DU145, $P<0.0001$), but no significant inhibition of cell proliferation was seen in *miR-187* or *miR-205* transfectants (Figures 3a and d).

A wound healing assay showed significant suppression of PC3 cell migration in *miR-222*- or *miR-31*-transfected PC3 cells compared with that of mock transfectants (the percentage of wound closure relative to mock was 47.6 ± 6.4% for *miR-222* transfectants, and 53.6 ± 16.6% for *miR-31* transfectants, $P<0.0001$), but no significant inhibition of wound closure was found in *miR-187*- or *miR-205*-transfectants in PC3 cells (Figure 3b). On the other hand, this assay showed that significant suppression of DU145 cell migration in *miR-205*, *miR-222*- or *miR-31*-transfected DU145 cells compared with that of mock transfectants (the percentage of wound closure relative to mock was 68.2 ± 16.9% for *miR-205* transfectants, 16.8 ± 13.4% for *miR-222* transfectants and 19.7 ± 11.6% for *miR-31* transfectants, $P<0.0001$) (Figure 3e). No significant inhibition of wound closure was found in *miR-187*-transfectants in DU145 cells.

A Matrigel invasion assay showed significant inhibition of PC3 cell invasion activity in *miR-222*- or *miR-31*- transfected PC3 cells compared with that of mock transfectants (the percentage of invasion cell number relative to mock was 4.4 ± 1.9% for *miR-222* transfectants and 8.4 ± 3.6% for *miR-31* transfectants, $P<0.0001$), but *miR-187* or *miR-205* transfectants showed no significant inhibition of cell invasion (Figure 3c). In DU145 cells, this assay showed that significant inhibition the cell invasion activity in *miR-205*, *miR-222* or *miR-31*- transfectants compared with that of mock transfectants (the percentage of invasion cell number relative to mock was 27.7 ± 3.7% for *miR-205* transfectants, 26.3 ± 9.4% for *miR-31* transfectants and 32.2 ± 6.7%, $P<0.0001$), but *miR-187* transfectant showed no significant inhibition of cell invasion (Figure 3f).

Search for candidate pathways of *miR-222* or *miR-31* by genome-wide gene expression and *in silico* analysis

We performed a genome-wide gene expression analysis of *miR-222* or *miR-31* transfected PC3 cells. In *miR-222* transfectants, 242 genes were downregulated (fold changes < -1.0) while *miR-31* transfectants had 694 genes downregulated compared with negative control miRNA transfectants. The current microarray data were approved by the GEO (Gene Expression Omnibus) and were assigned GEO accession number GSE37119. In this study, we have applied *in silico* analysis to identify the biological processes or pathways potentially regulated by *miR-222* or *miR-31*. These candidate target genes were assigned to pathways using GENECODIS software analysis (http://genecodis.cnb.csic.es), and statistically enriched pathways were identified. The GENECODIS software assigned a great many of the putative *miR-222* or *miR-31* targets to known pathways in KEGG, and these data facilitate the understanding of miRNA-regulated molecular pathways in human PCa cells. According to the GENECODIS software analysis, several pathways were identified, such as 'pathways in cancer', 'focal adhesion', and 'regulation of the actin cytoskeleton' (Table 3). We focused on the 'focal adhesion', and 'regulation of the actin cytoskeleton', as these were identified as enriched pathway both *miR-222* and *miR-31* target genes (Figures 4 and 5).

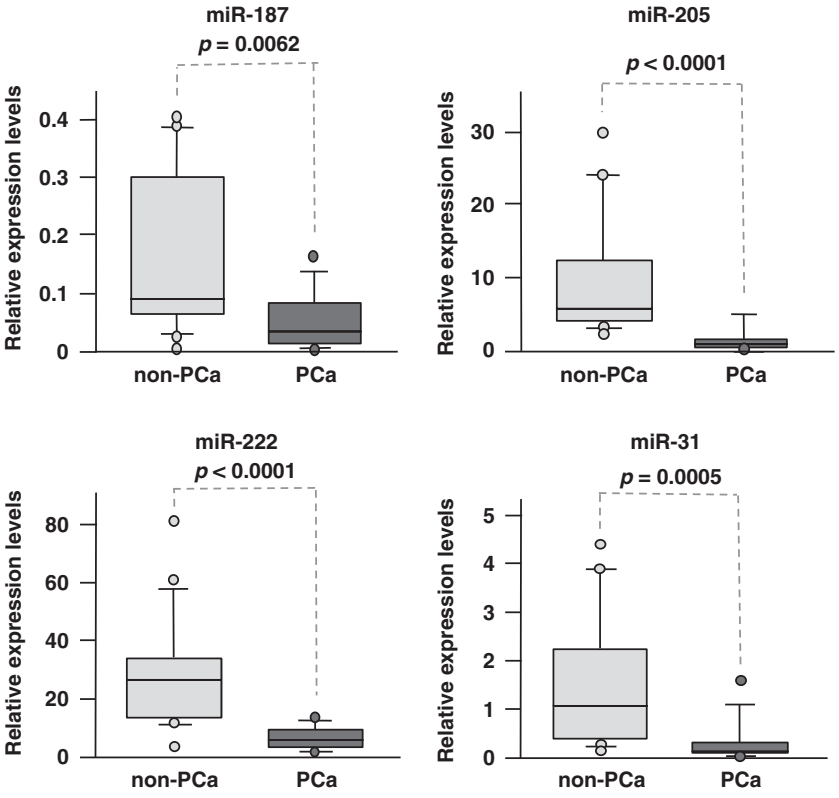

Figure 2 The expression levels of miRNAs (*miR-187*, *miR-205*, *miR-222* and *miR-31*) in clinical specimens. Real-time PCR showed that expression levels of four miRNAs were significantly lower in PCa than Non-PCa specimens. *RNU6B* was used as an internal control.

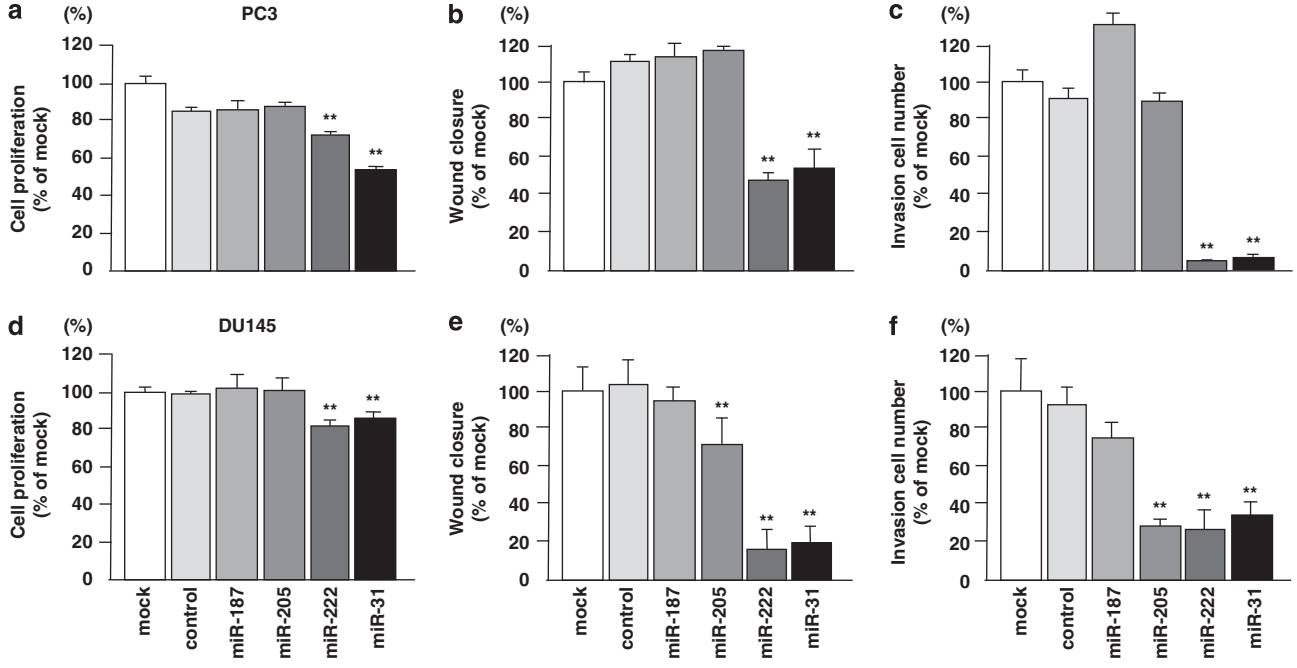

Figure 3 Effects of miRNA (*miR-187*, *miR-205*, *miR-222* and *miR-31*) transfection on PCa cell lines (PC3 and DU145). (**a**, **d**) Cell proliferation was determined by XTT assay in PCa cell lines 72 h after transfection of 10 nM of each miRNA and miRNA-control. The XTT assay showed significant inhibition of cell proliferation in *miR-222* and *miR-31* transfectant in comparison with mock cultures. (**b**, **e**) Cell migration activity determined by the wound healing assay and this assay showed significant inhibition of cell migration in *miR-222* and *miR-31* transfectants in comparison with mock cultures. (**c**, **f**) Cell invasion activity determined by the matrigel invasion assay and this assay showed significant inhibition of cell invasion in *miR-222* and *miR-31* transfectants in comparison with mock cultures. $^{**}P<0.001$.

Table 3 Top 10 pathways regulated by the putative targets of (a) miR-222 and (b) miR-31

KEGG pathway	Pathway ID	Found genes	P-value
(a) Top 10 pathways regulated by the putative targets of miR-222			
Regulation of actin cytoskeleton	hsa04810	8	0.00289864
Focal adhesion	hsa04510	7	0.00411682
Pathways in cancer	hsa05200	6	0.0354325
Protein processing in endoplasmic reticulum	hsa04141	5	0.0109403
Protein digestion and absorption	hsa04974	4	0.00971935
Valine, leucine and isoleucine degradation	hsa00280	4	0.003169
Jak-STAT signaling pathway	hsa04630	4	0.0355345
Fc gamma R-mediated phagocytosis	hsa04666	4	0.00844106
PPAR signaling pathway	hsa03320	3	0.0234498
Rheumatoid arthritis	hsa05323	3	0.0365932
(b) Top 10 pathways regulated by the putative targets of miR-31			
Pathways in cancer	hsa05200	19	0.000595902
Cell cycle	hsa04110	16	8.18E-07
Focal adhesion	hsa04510	15	0.000281747
Regulation of actin cytoskeleton	hsa04810	12	0.00496298
Axon guidance	hsa04360	12	0.000259021
Endocytosis	hsa04144	11	0.00785486
Lysosome	hsa04142	9	0.00333446
Protein processing in endoplasmic reticulum	hsa04141	9	0.0125215
Calcium signaling pathway	hsa04020	8	0.0398337
Oocyte meiosis	hsa04114	8	0.00641748

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; miR, micro RNA.

DISCUSSION

In this study we constructed miRNA expression signatures of clinical PCa specimens using 778 miRNAs by PCR-based analysis. Among these, 56 miRNAs were downregulated compared with non-PCa specimens (log2 ratio < -1.0 and $P<0.05$) and several miRNAs that we previously reported to be tumor suppressive in PCa, such as *miR-133a*, *miR-1* and *miR-145* were included in this signature.[15,17,18] Interestingly, *miR-1* and *miR-133a* have the same chromosomal locus in the human genome and *miR-1* and *miR-133a* downregulation is a frequent event in several cancers.[19] Our previous studies indicated that both miRNAs function as tumor suppressors that target multiple oncogenes.[12,20,21] Recent reports also indicated that *miR-1* acts as a tumor suppressor in PCa by influencing multiple cancer-related processes and inhibiting cell proliferation and motility.[22] We also reported *miR-145* downregulation in many types of human cancers, including PCa, suggesting that *miR-145* functions as a tumor suppressor. Indeed, *miR-145* is a widely accepted tumor suppressor in human cancers and targets various oncogenes in cancer cells.[17,18,23,24] The miRNA signature described here includes approved miRNAs that are downregulated in human cancers, including PCa, and indicates the effectiveness of this signature for cancer research.

Among the downregulated miRNAs present in this signature, the top four downregulated miRNAs (*miR-187*, *miR-205*, *miR-222* and *miR-31*) were evaluated to determine whether they had potential tumor suppressor functions (the fifth most downregulated miRNA, *miR-133a*, we previously reported to be a tumor suppressive miRNA in PCa). Our functional screening revealed that two miRNAs (*miR-31* and *miR-222*) significantly inhibited cell proliferation, migration and

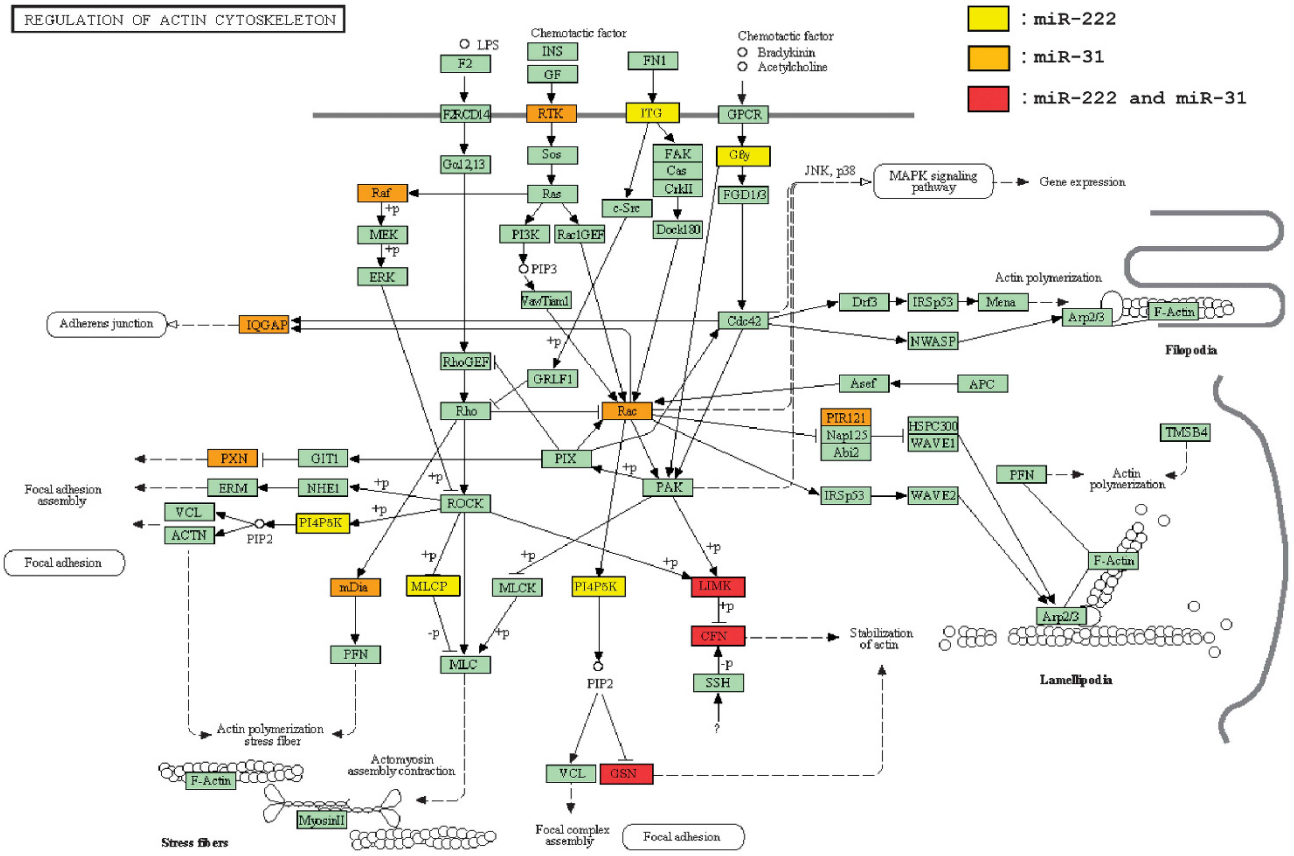

Figure 4 Putative *miR-222* and *miR-31* target genes in 'Regulation of actin cytoskeleton' from KEGG. The genes, putative *miR-222* target genes (yellow), *miR-31* (orange) and both *miR-222* and *miR-31* (red) as defined by the KEGG pathway and determined through GENECODIS analysis.

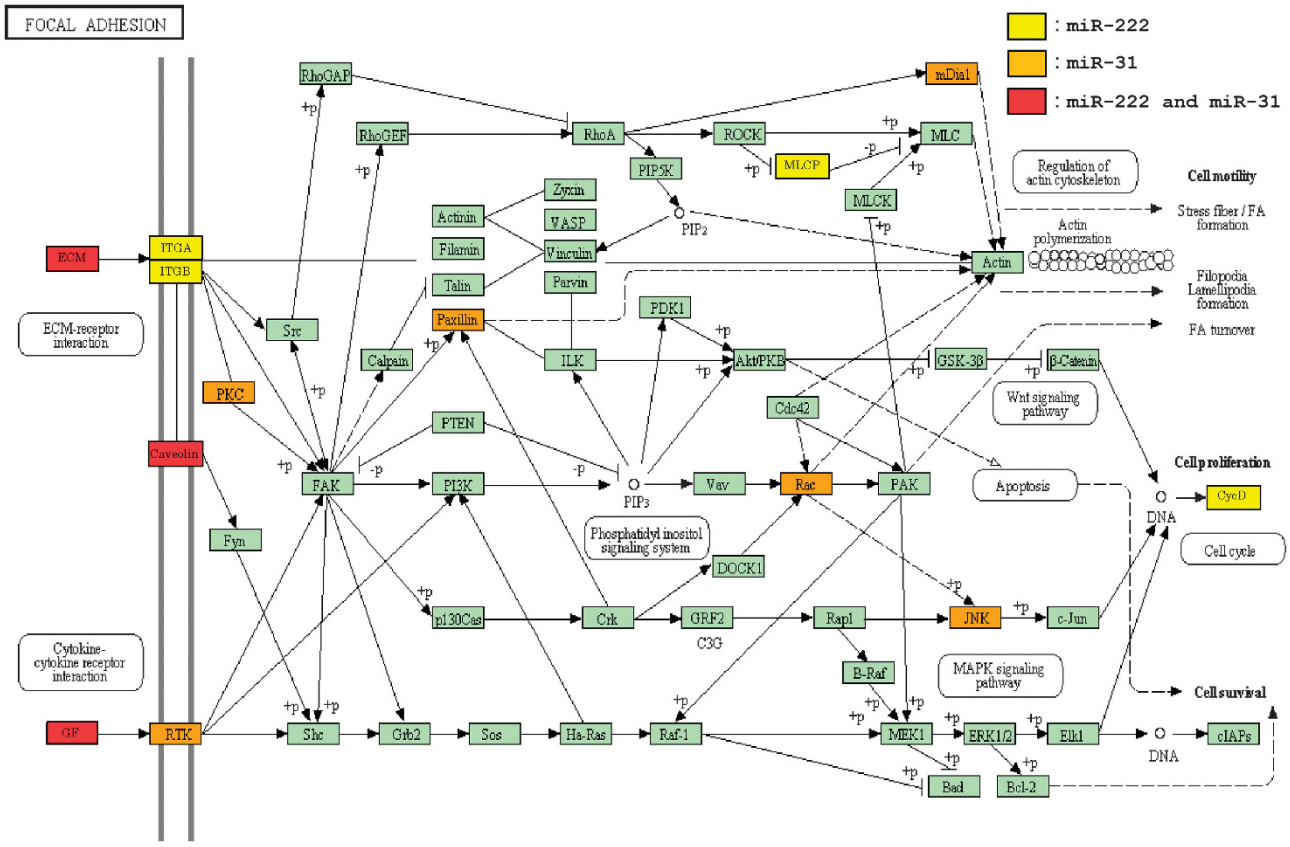

Figure 5 Putative *miR-222* and *miR-31* target genes in 'Focal adhesion' from KEGG. The genes, putative *miR-222* target genes (yellow), *miR-31* (orange) and both *miR-222* and *miR-31* (red) as defined by the KEGG pathway and determined through GENECODIS analysis.

invasion in PCa cell lines (PC3 and DU145) commonly, suggesting that these two miRNAs could be tumor suppressive miRNAs in PCa.

miR-222 is located close to *miR-221* and together they form a cluster on human chromosome X. *miR-222* has been reported to be upregulated in several types of human tumors, such as glioblastoma, non-small lung cancer and hepatocellular cancer. Regarding PCa, *miR-222* has been demonstrated to be highly expressed in androgen-independent PCa cells, compared with androgen-dependent PCa cells.[25,26] *miR-222* was also reported to reduce dihydrotestosterone-independent cell growth by directly targeting the cell cycle inhibitor p27/kip1, suggesting that *miR-222* might contribute to the development of castration-resistant PCa.[25] However, several articles also showed downregulation of *miR-222* in androgen-dependent PCa cells.[27,28] In the present study, *miR-222* was significantly downregulated in PCa specimens compared with non-PCa specimens and restoration of *miR-222* produced significant inhibition of proliferation, migration and invasion in PC3 and DU145 cells.

Downregulation of *miR-31* has been reported in various types of cancers, such as breast,[29] ovary,[30] stomach[31] and prostate.[32] Furthermore, homozygous loss of the *miR-31*-encoding genomic locus has been described in human urothelial carcinoma[33] and acute lymphoblastic lymphoma.[34] On the other hand, several studies showed that *miR-31* is upregulated in colorectal cancer, hepatocellular cancer and most squamous cell carcinomas. To date, considerable evidence has revealed the important roles of *miR-31* in cancer, but whether this miRNA functions as a tumor suppressor or an oncogene differs according to cancer type. *miR-31* reportedly contributes to apoptosis resistance in PCa cells by targeting the anti-apoptotic protein E2F6[29,35] and can antagonize metastasis by suppressing expression levels of ITGA5, RDX and RhoA in breast cancer.[36] Interestingly, our functional analyses showed that restoration of *miR-31* in PCa cell lines inhibited cell migration and invasion more significantly than cell proliferation, suggesting that *miR-31* may also contribute to cancer invasion and metastasis in PCa.

At present, several studies have investigated aberrant expression of miRNAs based on expression signatures in PCa samples.[27,37–39] Interestingly, our miRNA expression data were highly consistent with those of a previous study,[32] which found that *miR-205*, *miR-222* and *miR-31* were underexpressed in PCa. Genome-wide miRNA expression signatures can rapidly and precisely reveal aberrant expression of miRNA in cancers. The presence of common downregulated miRNAs on plural platforms provides important information for future analyses of PCa.

miRNAs are unique in their ability to regulate many protein coding genes with bioinformatic predictions indicating that miRNAs regulate more than 60% of protein coding genes.[6] The elucidation of new molecular pathways regulated by tumor suppressive *miR-222* or *miR-31* will be important for our understanding of PCa tumorigenesis. As such, we performed genome-wide gene expression and *in silico* analyses to search for molecular pathways regulated by *miR-222* or *miR-31*. However, as gene expression analysis can identify very large numbers of genes, to identify the biological processes or pathways potentially regulated by *miR-222* or *miR-31* in

PCa cells, we conducted a GeneCodis analysis[40] of our *miR-222* or *miR-31* transfectant expression profiles. The GeneCodis analysis applies many genes to known pathways in KEGG,[41] and these data facilitate understanding of tumor suppressive, miRNA-regulated molecular networks in cancer. According to the GeneCodis analysis, 'focal adhesion' and 'regulation of actin-cytoskeleton' categories were affected by both miRNA-regulated pathways. It is not easy to understand the functional significance of enormous genes which miRNA regulated in cancer cells. Like our current study, several groups reported that numerical target genes regulated by tumor suppressive miRNAs (*miR-222* or *miR-31*) were applied on the commonly accepted molecular pathways.[42–45] This approach provides new information and contributes to understand of the relation of human tumorigenesis and tumor suppressive miRNAs. In this study, both tumor suppressive *miR-222* and *miR-31* contributed to cancer cell migration and invasion and these pathways may participate in this phenomenon. Functional analysis of these pathways will be important for further understanding of PCa tumorigenesis, invasion and metastasis.

In conclusion, *miR-187*, *miR-205*, *miR-222* and *miR-31* were significantly downregulated in the PCa miRNA expression signature, with *miR-222* and *miR-31* functioning as tumor suppressors in PCa. The tumor suppressive, miRNA-regulated novel pathways in human PCa could provide new information concerning the molecular mechanisms of PCa tumorigenesis as well as tumor progression and metastasis.

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