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miRNA-93 in Serum Extracellular Vesicles Before and After Low Dose Rate Prostate Brachytherapy

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Abstract. *Background/Aim: To identify novel biomarkers for prostate cancer (PC), we evaluated changes of miRNAs contained in serum small extracellular vesicles (EVs) in patients who received low dose rate prostate brachytherapy (BT). Materials and Methods: EVs were isolated from the pooled serum of 10 PC patients prior to and 1 month after BT. miRNA profiling and quantitation in EVs was performed by microarray analysis and RT-digital PCR, respectively. Expression of miRNA-93 in prostate tissue was evaluated using the TCGA database and its level in EVs was determined in 25 patients before and 1, 3, 6 and 12 months after BT. Results: Profiling and quantitation identified miRNA-93 as significantly down-regulated in EVs after BT. TCGA database analysis showed that miRNA-93 was increased in PC tissue. miRNA-93 in EVs significantly decreased in 3, 6 and 12 months after BT. Conclusion: miRNA-93 contained in serum EVs may be a novel diagnostic and monitoring biomarker for PC.*

Prostate cancer (PC) is the most common male malignancy and the second leading cause of death in the United States, with 248,530 newly diagnosed cases and 34,130 deaths in 2020 (1). Prostate specific antigen (PSA) is the most widely utilized diagnostic and monitoring biomarker for PC. However, PSA has limitations as a marker owing to its low specificity (2). Novel biomarkers to accurately diagnose and monitor PC are mandatory.

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MicroRNAs (miRNAs) are short 18-24 nucleotide non-coding RNAs that regulate gene expression at the post transcriptional level (3, 4). miRNAs are present not only in cells but also in body fluids, known as circulating miRNAs (5). Circulating miRNAs are secreted from cells to the extracellular environment, either incorporated into the extracellular vesicles (EVs) or coupled with proteins such as argonaute 2 (Ago2), nucleophosmin 1 (NPM 1) and high density lipoprotein (HDL) (5). Thus, circulating miRNAs are associated with intracellular processes and are considered as less invasive biomarkers (6-8). Endzalins *et al*. have reported that miRNA signatures are clearly different between whole plasma and EVs (9). They showed that miRNA-200c-3p, miRNA-21-5p, miRNA-30c-5p and let-7a-5p in EVs but not in whole plasma were significantly up-regulated in PC compared to benign prostatic hyperplasia, suggesting that miRNAs in EVs provide more consistent results. However, EVs isolated by conventional methods including ultracentrifugation, polymeric precipitation and immunoprecipitation are heavily contaminated with serum proteins. Nakai *et al*. recently reported that highly-purified EVs can be isolated from serum by using T-cell immunoglobulin domains and mucin domain-containing protein 4 (Tim4) that binds to phosphatidylserine expressed on EVs (10). The use of highly-purified EVs isolated by the Tim4-based method may minimize the contamination with miRNAs bound to serum proteins and enable the analysis of miRNAs contained in EVs, leading to more accurate diagnosis. However, EVs are also secreted from various other cells in addition to PC cells. We hypothesized that we could identify PC-related miRNAs in EVs by comparing them before and after radical therapy.

Materials and Methods

Patients. This study was approved by the Medical Review Board of Gifu University, Graduate School of Medicine (# 29-410). Written informed consent was obtained from all patients. Serum was

collected from 10 PC patients before and 1 month after BT for microarray and quantitation analysis. To confirm miRNA expression changes, serum was also collected from 25 patients before and 1, 3, 6 and 12 months after BT including 10 patients whose serum was used for microarray analysis. Serum was sequentially centrifuged at 1,800 × *g* for 10 min to remove cells and at 16,500 × *g* for 20 min to remove debris and large EVs. Samples were then stored at –80˚C until further use.

Extracellular vesicle isolation. Isolation of EVs using the Tim4-based method has been previously described (11). Briefly, the biotinylated mouse Tim4/human Fc region of human IgG1 chimera recombinant protein (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was conjugated to streptavidin-magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA). Serum was incubated for 1 h at room temperature with Tim4-condjugated beads in the presence of 2 mM $CaCl_2$. After washing with TBS-T with 2 mM $CaCl_2$, captured EVs were released in 50 μl of TBS containing 2 mM EDTA. Isolated EVs were stored at -80 ˚C until further use.

Total RNA isolation. Total RNA in serum EVs was extracted using RNeasy Serum/Plasma kit (Qiagen, Venlo, the Netherlands). Briefly, EVs isolated from 1 ml of serum were lysed using QIAzol lysis solution. After mixing with chloroform and centrifugation at 12,000 × *g* for 15 min, total RNA was extracted from the supernatant using RNeasy MinElute spin column.

Microarray analysis. Two hundred μl of serum from 10 patients were mixed and EVs were isolated from total of 2 ml of pooled serum. Total RNA extracted from EVs was subjected to miRNA microarray analysis at TORAY Industries (Kamakura, Japan). Predicted pathways were analyzed based on the miRNA microarray data at TORAY Industries.

Reverse transcriptase-digital PCR. Two μl of RNA were subjected to cDNA synthesis using TaqMan Advanced miRNA assays according to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, poly(A) was attached to the tail of RNAs and then ligated. The ligation reaction was reverse transcribed to 50 μl of cDNA. Digital PCR was performed using 1.5 μl of the cDNA sample according to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, TaqMan for miRNA-5006, 6895, 4500 and 93 were purchased from Thermo Fisher Scientific. A mixture of cDNA and TaqMan reagents was loaded onto a 20,000-well chip and then PCR amplification was carried out with thermal cycling conditions of 96˚C (10 min), 60˚C (2 min), 98˚C (30 s, 39 cycles) and 60˚C (2 min). Absolute quantification was determined using the QuantStudio 3D digital PCR system (Thermo Fisher Scientific). The miRNA copy number contained in EVs from 1 ml of serum was then calculated.

miRNA-93 expression in prostate tissue using TCGA database. miRNA-93 sequence data among 497 PC and 52 normal prostate tissue (normal) were downloaded from The Cancer Genome Atlas (TCGA) database using the Genomic Data Commons Data Portal (https://gdc-portal.nci.gov/) and miRNA expression levels in primary PC and normal tissue were analyzed.

Statistical analysis. The student *t*-test was used to compare two parametric groups and the Mann-Whitney *U*-test for non-parametric groups. To compare changes of miRNA-93 expression before and after treatment, one-way ANOVA with Dunnett's test was used. The data analysis was performed using IBM SPSS Statistics version 23 software (Armonk, NY, USA). $p<0.05$ was considered statistically significant.

Results

miRNA microarray analysis. We performed microarray analysis of miRNAs contained in EVs isolated by the Tim4-based method from pooled serum of 10 PC patients before and 1 month after BT and compared the expression changes. miRNAs with a 2-fold or greater expression change are listed in Tables I and II. The top 10 relevant pathways associated with up- and down-regulated miRNAs are listed in Table III. The PI3K/AKT signaling pathway was found to be the most relevant pathway associated with both up- and down-regulated miRNAs.

Confirmation of miRNA expression changes. Among miRNAs identified as up- and down-regulated by microarray analysis, we performed RT-digital PCR for miRNA-5006, –6895, –4500 and –93 using EVs isolated from serum of 10 patients from which pooled serum was prepared for microarray analysis. The level of miRNA-93 in EVs was significantly down-regulated after BT (0.11 fold, $p=0.034$), while other 3 miRNAs did not change (Figure 1).

miRNA-93 expression levels in primary tumors and normal prostate tissue. Since miRNA-93 was found to be decreased in serum EVs after BT, we examined if its expression was elevated in PC tissue using the TCGA database. The analysis showed that miRNA-93 was significantly up-regulated in primary PC tissue compared with normal tissue (3.44 fold, $p<0.001$, Figure 2).

Changes of miRNA-93 expression in serum EVs after BT. We examined the changes of miRNA-93 expression in serum EVs repeatedly after BT in 25 PC patients to evaluate its potential as a monitoring marker. miRNA-93 was significantly decreased at 3, 6 and 12 months after BT ($p=0.018$, 0.002 and 0.027, respectively, Figure 3).

Discussion

In this study, we evaluated miRNA changes in serum EVs after BT to identify novel diagnostic and monitoring biomarkers for PC. According to the results of microarray analysis followed by confirmation analysis with RT-dPCR, we found that miRNA-93 contained in EVs was significantly down-regulated 1 month after BT (Table I, Figure 1), suggesting that the miRNA-93 change in EVs may be related to PC. miRNA-93 has been reported to be an oncogenic miRNA that plays pivotal roles in invasion, migration, proliferation, cell growth and progression among various

Table I. *miRNAs in serum extracellular vesicles up-regulated by 2-fold or greater after low dose rate prostate brachytherapy.*

Name	Ratio	Name	Ratio	Name	Ratio	Name	Ratio
hsa-miR-5006-3p	84.93	hsa-miR-1256	9.01	hsa-miR-152-3p	6.10	hsa-miR-191-3p	2.20
hsa-miR-519c-5p	30.74	hsa-miR-4668-3p	8.99	hsa-miR-26a-1-3p	6.06	hsa-miR-518f-3p	2.18
hsa-miR-523-5p	30.74	hsa-miR-4302	8.85	hsa-miR-323a-3p	5.09	hsa-miR-367-5p	2.11
hsa-miR-518e-5p	30.74	hsa-miR-4652-3p	8.76	hsa-miR-4669	4.93	hsa-miR-4767	2.10
hsa-miR-522-5p	30.74	hsa-miR-23c	8.73	hsa-miR-5571-5p	3.77	hsa-miR-6872-3p	2.07
hsa-miR-519a-5p	30.74	hsa-miR-6875-3p	8.72	hsa-miR-4697-3p	3.71	hsa-miR-4454	2.07
hsa-miR-519b-5p	30.74	hsa-miR-597-5p	8.71	hsa-miR-6835-3p	3.57	hsa-miR-151a-5p	2.03
hsa-miR-941	20.19	hsa-miR-19a-5p	8.50	hsa-miR-4745-3p	3.42	hsa-miR-17-3p	2.03
hsa-miR-518e-3p	19.43	hsa-miR-5587-3p	8.26	hsa-miR-3181	3.40	hsa-miR-4286	2.00
hsa-miR-647	19.29	hsa-miR-1283	8.22	hsa-miR-4313	3.29		
hsa-miR-3142	18.58	hsa-miR-135b-3p	8.21	hsa-miR-181b-2-3p	3.22		
hsa-miR-6895-3p	15.54	hsa-miR-33b-5p	8.17	hsa-miR-4292	3.19		
hsa-miR-1245a	14.87	hsa-miR-6841-3p	8.06	hsa-miR-128-3p	3.18		
hsa-miR-518d-3p	14.16	hsa-miR-5092	7.81	hsa-miR-1267	3.14		
hsa-miR-548d-3p	14.05	hsa-miR-4790-5p	7.77	hsa-miR-339-5p	3.06		
hsa-miR-4480	13.19	hsa-miR-596	7.74	hsa-miR-345-5p	3.00		
hsa-miR-2115-5p	13.15	hsa-miR-4285	7.72	hsa-miR-6840-5p	3.00		
hsa-miR-548o-3p	12.49	hsa-miR-153-3p	7.61	hsa-miR-195-3p	2.86		
hsa-miR-1296-5p	12.28	hsa-miR-6746-3p	7.60	hsa-miR-4757-5p	2.76		
hsa-miR-523-3p	12.22	hsa-miR-200a-5p	7.51	hsa-miR-939-3p	2.69		
hsa-miR-448	12.14	hsa-miR-192-3p	7.44	hsa-miR-365a-3p	2.66		
hsa-miR-331-3p	12.12	hsa-miR-5691	7.28	hsa-miR-365b-3p	2.66		
hsa-miR-4757-3p	11.64	hsa-miR-193a-3p	7.25	hsa-miR-1323	2.53		
hsa-miR-542-5p	11.63	hsa-miR-380-5p	7.19	hsa-miR-516a-5p	2.48		
hsa-miR-934	11.41	hsa-miR-4714-5p	7.01	hsa-miR-6070	2.47		
hsa-miR-132-3p	11.20	hsa-miR-4287	6.98	hsa-miR-933	2.46		
hsa-miR-662	11.17	hsa-miR-186-5p	6.97	hsa-miR-425-3p	2.42		
hsa-miR-4727-5p	11.12	hsa-miR-586	6.96	hsa-miR-324-5p	2.42		
hsa-miR-29c-3p	10.64	hsa-miR-216a-5p	6.94	hsa-miR-106a-3p	2.41		
hsa-miR-2115-3p	10.50	hsa-miR-4663	6.93	hsa-miR-885-5p	2.39		
hsa-miR-548g-3p	10.01	hsa-miR-4785	6.84	hsa-miR-1251-5p	2.35		
hsa-miR-1248	9.89	hsa-miR-1273c	6.77	hsa-miR-5100	2.34		
hsa-miR-520a-5p	9.80	hsa-miR-7849-3p	6.77	hsa-miR-99a-3p	2.32		
hsa-miR-548ay-3p	9.73	hsa-miR-548p	6.70	hsa-let-7f-1-3p	2.32		
hsa-miR-127-3p	9.67	hsa-let-7i-3p	6.37	hsa-miR-504-5p	2.32		
hsa-miR-500a-3p	9.62	hsa-miR-577	6.37	hsa-let-7e-3p	2.29		
hsa-miR-6890-3p	9.62	hsa-miR-6807-3p	6.37	hsa-miR-6752-3p	2.29		
hsa-miR-6720-5p	9.60	hsa-miR-891a-5p	6.37	hsa-miR-31-3p	2.26		
hsa-miR-526b-3p	9.47	hsa-miR-576-5p	6.29	hsa-miR-133a-3p	2.22		
hsa-miR-202-5p	9.06	hsa-miR-137	6.22	hsa-miR-518c-3p	2.22		

The ratio represents miRNA levels after treatment divided by those before treatment.

cancers (12-15). In PC cells, miRNA-93 has also been shown to be involved in cell proliferation, invasion, migration and apoptosis and its high expression correlated with a poor prognosis among PC patients (16-19). It has been shown that miRNA-93 is significantly up-regulated in PC tissues compared to normal prostate tissues (20, 21). Using the TCGA database, we also confirmed that miRNA-93 was elevated in PC tissue compared with normal prostate tissue. These findings suggest that miRNA-93 overexpression in prostate tissue could be a biomarker for PC.

To evaluate miRNA-93 expression in prostate tissue, prostate biopsy is essential, but this is an invasive process, unsuitable for PC screening. Circulating miRNAs have been reported as a less invasive marker to diagnose PC (9, 20-22) and circulating miRNA-93 was shown to be a potential diagnostic marker for PC and be associated with tumor aggressiveness (20, 21). Circulating miRNA-93 includes both EV-contained and protein-bound miRNA-93. In the present study, we identified miRNA-93 as a marker for PC, in highly-purified EVs isolated by the Tim4-based method with minimal contamination with miRNAs bound to serum proteins. Zedan *et al*. reported that circulating miRNA-93 was down-regulated 6 months after radical prostatectomy and radiation therapy for PC (23), but, to the best of our

Table II. *miRNAs contained in extracellular vesicles down-regulated by 2-fold or greater after low dose rate brachytherapy.*

Name	Ratio	Name	Ratio	Name	Ratio	Name	Ratio	Name	Ratio	Name	Ratio	Name	Ratio
hsa-miR-4500	0.01	hsa-miR-4306	0.14	hsa-miR-6769b-3p	0.19	hsa-miR-28-5p	0.22	hsa-miR-199b-5p	0.27	hsa-miR-7856-5p	0.32	hsa-miR-185-5p	0.44
hsa-miR-15a-5p	0.04	hsa-miR-6820-3p	0.14	hsa-miR-5187-5p	0.19	hsa-miR-6750-3p	0.22	hsa-let-7b-3p	0.27	hsa-miR-512-5p	0.32	hsa-miR-7162-3p	0.44
hsa-miR-195-5p	0.06	hsa-miR-5004-5p	0.14	hsa-miR-7110-3p	0.19	hsa-miR-4733-5p	0.23	hsa-miR-526b-5p	0.27	hsa-miR-619-5p	0.32	hsa-miR-6718-5p	0.44
hsa-miR-93-5p	0.06	hsa-miR-6734-5p	0.14	hsa-miR-3127-3p	0.19	hsa-miR-18b-5p	0.23	hsa-miR-6868-5p	0.27	hsa-miR-1246	0.32	hsa-miR-6751-5p	0.45
hsa-miR-3692-5p	0.06	hsa-let-7i-5p	0.14	hsa-miR-5681a	0.19	hsa-miR-3156-3p	0.23	hsa-miR-3150a-5p	0.27	hsa-miR-323b-3p	0.32	hsa-miR-7154-3p	0.45
hsa-miR-144-3p	0.07	hsa-miR-6852-5p	0.14	hsa-miR-4646-3p	0.19	hsa-miR-1825	0.23	hsa-miR-520g-3p	0.27	hsa-miR-4761-5p	0.32	hsa-miR-20b-5p	0.45
hsa-miR-4289	0.07	hsa-miR-3127-5p	0.14	hsa-miR-129-5p	0.19	hsa-miR-4786-3p	0.23	hsa-miR-642a-5p	0.27	hsa-miR-6511a-3p	0.32	hsa-let-7f-5p	0.45
hsa-let-7g-5p	0.09	hsa-miR-3667-5p	0.15	hsa-miR-632	0.19	hsa-miR-6791-3p	0.23	hsa-miR-4502	0.27	hsa-miR-1911-3p	0.32	hsa-miR-4779	0.45
hsa-miR-6783-5p	0.09	hsa-miR-4441	0.15	hsa-miR-142-3p	0.19	hsa-miR-146b-5p	0.23	hsa-miR-4455	0.28	hsa-miR-20a-5p	0.33	hsa-miR-1286	0.45
hsa-miR-3689a-3p	0.09	hsa-miR-30a-5p	0.15	hsa-miR-6863	0.20	hsa-miR-25-3p	0.23	hsa-miR-4694-3p	0.28	hsa-miR-26a-2-3p	0.33	hsa-miR-19b-3p	0.45
hsa-miR-6770-5p	0.10	hsa-miR-6853-5p	0.15	hsa-miR-6770-3p	0.20	hsa-miR-4685-3p	0.23	hsa-miR-639	0.28	hsa-miR-18a-5p	0.33	hsa-miR-378b	0.45
hsa-miR-3622a-3p	0.10	hsa-miR-485-5p	0.15	hsa-miR-3972	0.20	hsa-miR-140-3p	0.23	hsa-miR-1207-3p	0.28	hsa-miR-548az-3p	0.33	hsa-miR-139-3p	0.46
hsa-miR-5091	0.10	hsa-miR-6503-3p	0.15	hsa-miR-3155b	0.20	hsa-miR-517-5p	0.23	hsa-miR-410-3p	0.28	hsa-miR-6769a-3p	0.33	hsa-miR-208a-5p	0.47
hsa-miR-4311	0.10	hsa-miR-7151-3p	0.15	hsa-miR-6734-3p	0.20	hsa-miR-4268	0.23	hsa-miR-6761-3p	0.28	hsa-miR-3116	0.33	hsa-miR-103a-3p	0.48
hsa-miR-7113-5p	0.10	hsa-miR-938	0.16	hsa-miR-199a-5p	0.20	hsa-miR-8485	0.23	hsa-miR-34c-3p	0.28	hsa-miR-4726-3p	0.33		
hsa-miR-378a-3p	0.11	hsa-miR-151b	0.16	hsa-miR-4296	0.20	hsa-miR-489-3p	0.23	hsa-miR-125a-5p	0.28	hsa-miR-496	0.33		
hsa-miR-6870-3p	0.11	hsa-miR-4479	0.16	hsa-miR-204-5p	0.20	hsa-miR-6889-3p	0.23	hsa-miR-3189-3p	0.28	hsa-miR-4474-3p	0.34		
hsa-miR-106b-5p	0.11	hsa-miR-6877-3p	0.16	hsa-miR-6084	0.20	hsa-miR-432-3p	0.23	hsa-miR-155-5p	0.28	hsa-miR-4290	0.34		
hsa-miR-574-5p	0.11	hsa-miR-222-3p	0.16	hsa-miR-4647	0.20	hsa-miR-1208	0.24	hsa-miR-93-3p	0.29	hsa-miR-424-3p	0.35		
hsa-miR-4439	0.11	hsa-miR-6816-3p	0.16	hsa-miR-1273h-3p	0.20	hsa-miR-648	0.24	hsa-miR-500b-3p	0.29	hsa-miR-1276	0.35		
hsa-miR-6723-5p	0.12	hsa-miR-8075	0.16	hsa-miR-323b-5p	0.20	hsa-miR-129-2-3p	0.24	hsa-miR-16-5p	0.29	hsa-miR-1306-3p	0.35		
hsa-miR-320c	0.12	hsa-miR-6133	0.16	hsa-miR-566	0.20	hsa-miR-486-5p	0.24	hsa-miR-1285-3p	0.29	hsa-miR-4733-3p	0.35		
hsa-miR-492	0.12	hsa-miR-3120-3p	0.16	hsa-miR-2909	0.20	hsa-miR-4804-5p	0.24	hsa-miR-7161-3p	0.29	hsa-miR-339-3p	0.35		
hsa-miR-4773	0.12	hsa-miR-2277-3p	0.17	hsa-miR-3690	0.20	hsa-miR-6826-3p	0.24	hsa-miR-5010-3p	0.29	hsa-miR-6787-3p	0.35		
hsa-miR-758-5p	0.12	hsa-miR-6779-3p	0.17	hsa-miR-5196-3p	0.21	hsa-miR-8082	0.25	hsa-miR-4746-5p	0.29	hsa-miR-664b-5p	0.35		
hsa-miR-30c-5p	0.12	hsa-miR-134-3p	0.17	hsa-miR-3691-5p	0.21	hsa-miR-1299	0.25	hsa-miR-6823-3p	0.30	hsa-miR-4653-3p	0.35		
hsa-miR-4737	0.12	hsa-miR-451a	0.17	hsa-miR-3150b-3p	0.21	hsa-miR-1303	0.25	hsa-miR-548x-3p	0.30	hsa-miR-4650-3p	0.35		
hsa-miR-6893-3p	0.12	hsa-miR-6735-3p	0.17	hsa-miR-6846-3p	0.21	hsa-miR-6757-3p	0.25	hsa-miR-4708-5p	0.30	hsa-miR-4534	0.36		
hsa-miR-4691-5p	0.12	hsa-miR-3612	0.17	hsa-miR-6832-3p	0.21	hsa-miR-3179	0.25	hsa-miR-610	0.30	hsa-miR-6823-5p	0.36		
hsa-miR-519d-3p	0.13	hsa-miR-3182	0.17	hsa-miR-219b-5p	0.21	hsa-miR-4263	0.25	hsa-miR-887-5p	0.30	hsa-let-7c-5p	0.37		
hsa-miR-4321	0.13	hsa-miR-6726-3p	0.17	hsa-miR-143-5p	0.21	hsa-miR-4633-5p	0.25	hsa-miR-2114-3p	0.30	hsa-miR-2467-5p	0.37		
hsa-miR-6715b-3p	0.13	hsa-miR-4425	0.17	hsa-miR-675-3p	0.21	hsa-miR-6886-3p	0.25	hsa-miR-6508-5p	0.30	hsa-miR-708-5p	0.37		
hsa-miR-615-3p	0.13	hsa-miR-6829-3p	0.18	hsa-miR-6737-3p	0.21	hsa-miR-432-5p	0.25	hsa-miR-337-3p	0.31	hsa-miR-6736-3p	0.37		
hsa-miR-1199-3p	0.13	hsa-miR-6817-5p	0.18	hsa-miR-6074	0.21	hsa-miR-193b-3p	0.25	hsa-miR-378e	0.31	hsa-miR-106a-5p	0.38		
hsa-miR-194-3p	0.13	hsa-miR-4421	0.18	hsa-miR-4755-3p	0.21	hsa-miR-138-5p	0.25	hsa-miR-6794-3p	0.31	hsa-miR-15b-5p	0.40		
hsa-miR-99b-3p	0.13	hsa-miR-6862-3p	0.18	hsa-miR-4276	0.21	hsa-let-7a-2-3p	0.26	hsa-miR-3121-3p	0.31	hsa-miR-761	0.41		
hsa-miR-6862-5p	0.13	hsa-miR-3654	0.18	hsa-miR-664a-3p	0.21	hsa-miR-598-5p	0.26	hsa-miR-4780	0.31	hsa-let-7d-5p	0.42		
hsa-miR-6809-5p	0.13	hsa-miR-5188	0.18	hsa-miR-21-3p	0.22	hsa-miR-4251	0.26	hsa-miR-376b-3p	0.31	hsa-miR-3130-3p	0.42		
hsa-miR-6759-3p	0.13	hsa-miR-516b-5p	0.19	hsa-miR-190a-3p	0.22	hsa-miR-524-3p	0.26	hsa-miR-181a-3p	0.32	hsa-miR-17-5p	0.42		
hsa-miR-142-5p	0.14	hsa-miR-485-3p	0.19	hsa-miR-155-3p	0.22	hsa-miR-1321	0.26	hsa-miR-3659	0.32	hsa-miR-6758-5p	0.43		

The ratio represents miRNA levels after treatment divided by those before treatment.

Table III. *Predicted pathways associated with up- or down-regulated miRNA changes after low dose rate prostate brachytherapy.*

Up-regulated		Down-regulated	
Pathway	*p*-Value	Pathway	*p*-Value
PI3K-Akt signaling pathway	5.31E-19	PI3K-Akt signaling pathway	5.73E-43
Nuclear receptors meta-pathway	5.31E-19	Focal adhesion-PI3K-Akt-mTOR-signaling pathway	2.12E-41
Focal adhesion-PI3K-Akt-mTOR-signaling pathway	2.00E-14	Nuclear receptors meta-pathway	2.89E-38
VEGFA-VEGFR2 signaling pathway	2.00E-14	GPCRs, Class A Rhodopsin-like	2.89E-38
Focal adhesion	3.88E-12	Insulin signaling	1.07E-36
Epithelial to mesenchymal transition in colorectal cancer	3.88E-12	VEGFA-VEGFR2 signaling pathway	5.34E-32
Wnt Signaling pathway and pluripotency	3.88E-12	EGF-EGFR signaling pathway	1.97E-30
Exercise-induced circadian regulation	3.88E-12	Breast cancer pathway	1.97E-30
Circadian rhythm related genes	3.88E-12	MAPK signaling pathway	1.97E-30
MAPK signaling pathway	3.88E-12	Angiopoietin like protein 8 regulatory pathway	1.97E-30

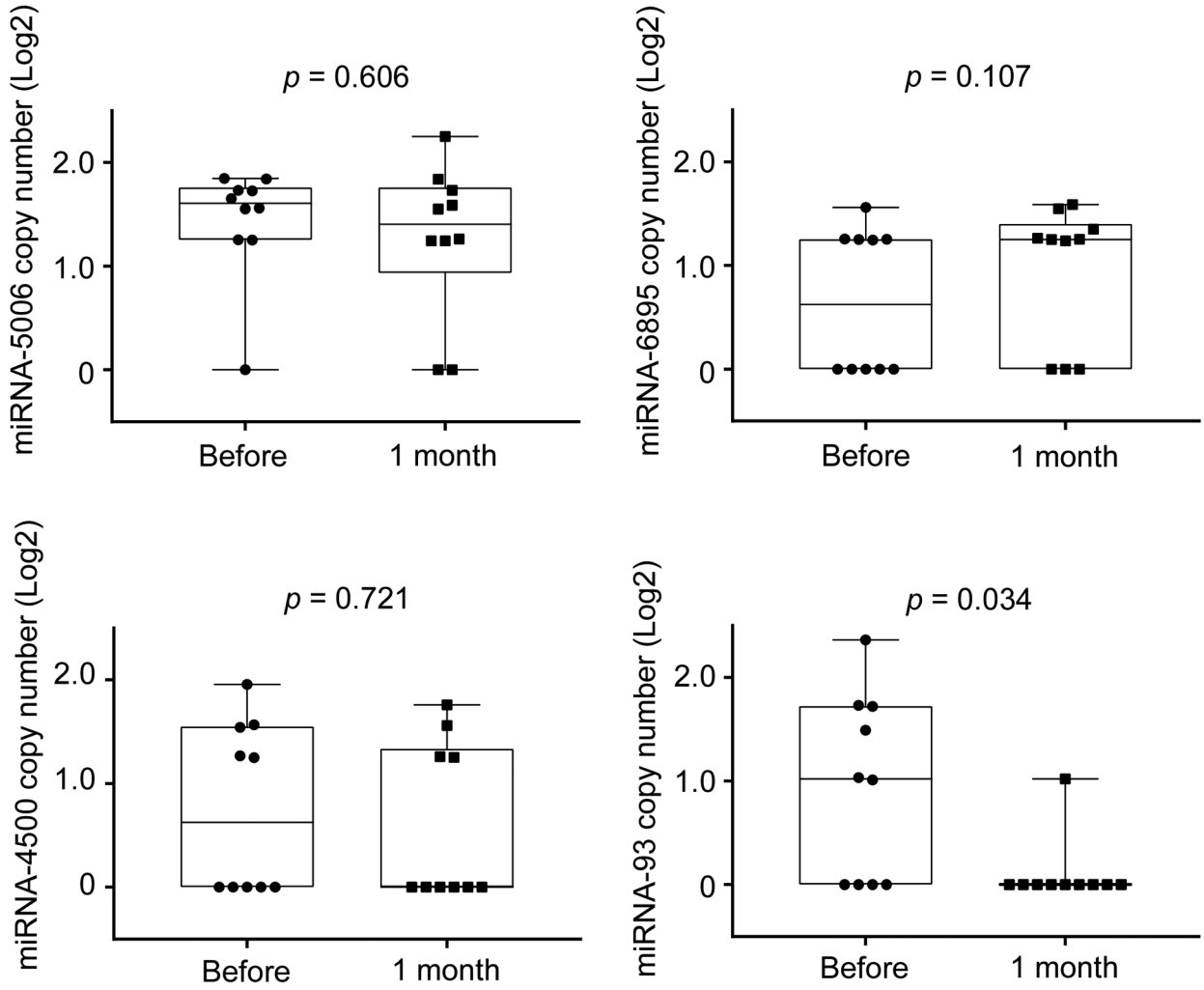

Figure 1. *Confirmation of miRNA changes in serum EVs 1 month after BT. miRNA-93 contained in serum EVs was significantly down-regulated after BT (0.11-fold, p=0.034), while miRNA-5006 (0.88-fold, p=0.606), miRNA-6895 (1.45-fold, p=0.107) and miRNA-4500 (0.77-fold, p=0.721) did not change significantly. The Student's t-test was used for comparison. The median is represented by horizontal bars. miRNA copy numbers are expressed in log scale.*

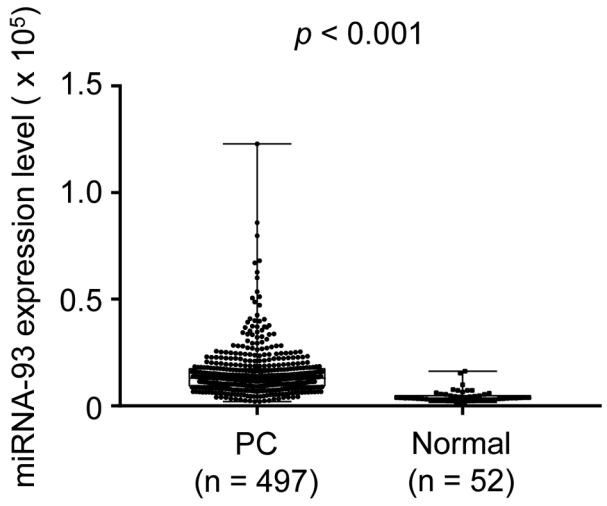

Figure 2. *miRNA-93 expression level in prostate tissue. miRNA-93 sequence data were obtained from TCGA. miRNA-93 was significantly up-regulated in PC tissue compared to normal tissue (3.44-fold, p<0.001, Mann-Whitney U-test).*

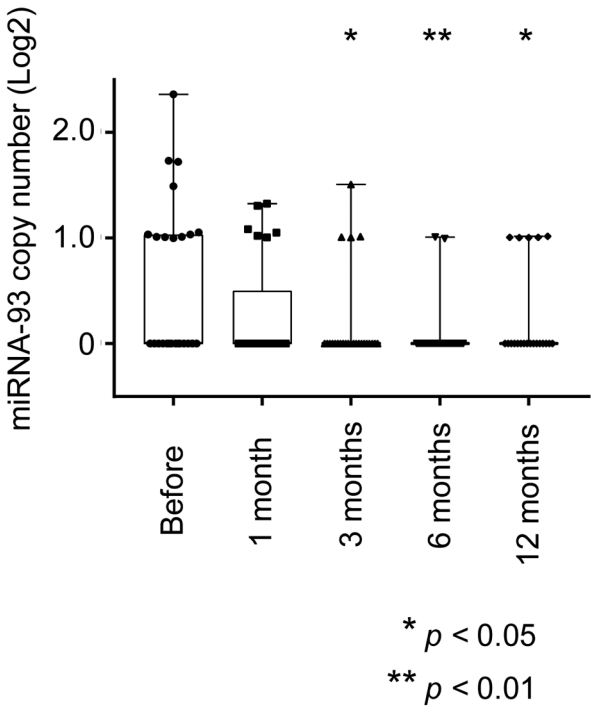

Figure 3. *Changes of miRNA-93 levels in serum EVs after BT. miRNA-93 was decreased at 1 month (0.47-fold, p=0.096), and significantly decreased at 3 months (0.31-fold, p=0.018), 6 months (0.14-fold, p=0.002) and 12 months (0.35-fold, p=0.027) after BT versus before treatment. One-way ANOVA followed by Dunnett's test was used to compare miRNA-93 changes. miRNA-93 copy numbers were expressed in log scale.*

knowledge, there have been no reports that measured circulating miRNA-93 levels repeatedly after radical therapy. We evaluated the levels of miRNA-93 in serum EVs at 1, 3, 6 and 12 months after BT to evaluate its potential as a monitoring marker. The results showed that the levels in serum EVs were significantly decreased at 3 months after BT and remained statistically significant at 6 and 12 months, suggesting that a decrease in miRNA-93 in EVs is likely related to PC and could be a monitoring marker for this type of cancer. In 5 out of 25 patients, miRNA-93 was positive at 12 months after BT. An increase in miRNA-93 in EVs could serve as a marker for recurrence after BT.

Based on the microarray analysis, we identified PI3K/AKT signaling as the most relevant pathway associated with both up- and down-regulated miRNAs after BT. PTEN is a key suppressor of PI3K/AKT signaling and its loss is commonly observed in aggressive PC patients such as castration-resistant and metastatic PC (24). Recently, several clinical trials targeting PTEN/PI3K/AKT/mTOR signaling are ongoing in PC patients (24-29). miRNA-93 was reported to suppress PTEN and activate the PI3K/AKT cascade in ovarian and breast cancer (30, 31). In this regard, therapy targeting PI3K/AKT signaling may be effective for miRNA-93-positive PC patients recurrent after BT.

We are aware of limitations of this study. This study included a small sample size and a short follow-up period. Large-scale analysis and evaluation of long term prognosis will be needed in the future.

In conclusion, we identified that miRNA-93 contained in highly-purified serum EVs could be a novel biomarker to diagnose and monitor PC and possibly to predict recurrence after BT.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

KI, KK and TKa designed the study and experiments. KI, KK, KM and TKa performed the experiments. KI, KK, KM, YF, Masafumi I and TKa analyzed data. KI, TKa, MN, TY, Masaya I and TKu performed BT. The first draft of the manuscript was written by KI, Masafumi I and TKa. All Authors read, commented on and approved the manuscript.

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