

Exosomal *microRNA-141* is upregulated in the serum of prostate cancer patients

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Purpose: Novel biomarkers for the diagnosis of prostate cancer (PCa) are urgently required. Increasing evidence suggests that exosomal microRNAs (miRNAs or miRs) in serum may be potential noninvasive biomarkers for certain diseases. The objective of the present study was to investigate and assess whether exosomal *miR-141* is an effective biomarker for human PCa.

Methods: In the present study, exosomes were isolated from the serum of patients with PCa, patients with benign prostate hyperplasia (BPH), and healthy volunteers. The total RNA was extracted from the exosomes and the level of *miR-141* was analyzed by quantitative reverse transcription-polymerase chain reaction. The expression levels of *miR-141* were compared between the whole serum and the serum exosomes of the three groups. Subsequently, the relevance of the exosomal expression of *miR-141* to the clinicopathological factors in PCa was investigated.

Results: The expression of *miR-141* was higher in exosomes compared with whole serum (control group, $P=0.0003$; BPH group, $P=0.0016$; PCa group, $P<0.0001$). The level of serum exosomal *miR-141* was significantly higher in the patients with PCa compared with the patients with BPH and the healthy controls (3.85-fold, $P=0.0007$ and 4.06-fold, $P=0.0005$, respectively). In addition, the expression levels were significantly higher in metastatic PCa compared with localized PCa ($P<0.0001$). Receiver-operating characteristic curve revealed that the serum exosomal *miR-141* yielded an area under the curve of 0.8694, with 80% sensitivity and 87.1% specificity in discriminating patients with metastatic PCa from the patients with localized PCa.

Conclusion: Serum exosomes may serve as a more suitable material compared with the whole serum for measuring circulating *miR-141* levels in patients with PCa. Exosomal *miR-141* is upregulated in the serum from patients with PCa compared with patients with BPH or the healthy volunteers, and it may be a useful potential biomarker for the diagnosis of metastatic PCa.

Keywords: exosomes, *microRNA-141*, serum, prostate cancer, biomarker

Introduction

Prostate cancer (PCa) is one of the most common male malignancies and the second leading cause of male cancer-associated mortality.¹ In the People's Republic of China, the incidence and mortality rates of PCa are clearly increasing. In addition, Chinese patients with PCa exhibit a higher proportion (13.3%–26%) of bone metastasis in the initial diagnosis.^{2–4} Currently, prostate-specific antigen (PSA) is the commonly used biomarker for PCa, which is measured in the blood and exhibits increased levels in patients with PCa. However, limitations of PSA screening usually lead to overdiagnosis and overtreatment.⁵ Therefore, novel biomarkers with increased specificity for the diagnosis and prognosis of PCa are urgently required. Increasing evidence suggests that exosomal microRNAs (miRNAs or miRs) in circulating fluids may offer an attractive potential as noninvasive biomarkers for certain diseases, including cancer.^{6–8}

Exosomes are 30–100 nm membrane vesicles, which are released from numerous cell types into the extracellular space. Exosomes are widely distributed in the

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blood, urine, and other bodily fluids. Lipids and proteins are the predominant components of exosomal membranes.⁹ Exosomes are important in the exchange of information between cells.¹⁰ Previously, miRNAs have been identified in exosomes, which can be taken up by neighboring or distant cells, and subsequently modulate the recipient cells.¹¹ Additionally, several previous studies have revealed that stable miRNAs can be detected in exosomes, as well as in serum, and the majority of serum-circulating miRNAs are enriched in exosomes. Furthermore, the quantity and composition of exosomal miRNAs are different between patients and healthy individuals.^{12,13} Therefore, exosomal miRNAs may serve as valuable noninvasive biomarkers for the diagnosis and prognosis of certain diseases.

However, a number of previous studies addressing the expression levels of circulating miRNAs in clinical patients with PCa were performed on total serum or plasma samples.^{14–17} With regards to exosomal miRNAs, to the best of our knowledge, only a few previous studies have reported the use of exosomal miRNAs for prognosis in patients with PCa.¹⁸ In addition, whether there is a potential benefit in using miRNAs in exosomes compared with in the total serum as biomarkers for patients with PCa remains to be elucidated.

miR-141 is one of the most common cancer-associated miRNAs, which has been reported as tumor suppressor in several malignancies, such as gastric cancer,^{19,20} pancreatic cancer,²¹ breast cancer,^{22,23} renal cell carcinoma,^{24,25} hepatocellular carcinoma,²⁶ and so on. On the contrary, increasing evidence shows that *miR-141* is upregulated in PCa, which is correlated with a high Gleason score or metastasis of patients.^{14,27–30} Therefore, *miR-141* is considered as a potential miRNA biomarker for PCa. However, the comparison of the expression levels of *miR-141* in serum samples and exosomes

isolated from the serum of patients with PCa remains to be elucidated. In the present study, the expression levels of *miR-141* in exosomes isolated from the serum samples of patients with PCa, benign prostate hyperplasia (BPH), and healthy volunteers were investigated. Furthermore, the potential clinical value of using exosomal *miR-141* as a biomarker for the detection of PCa was assessed.

Materials and methods

Patients and blood samples

Patients initially diagnosed with PCa (no prior treatment) at Xijing Hospital of Fourth Military Medical University (Xi'an, People's Republic of China) between January 2013 and December 2014 were included in the PCa group. Patients with newly diagnosed BPH (no prior treatment) were enrolled in the BPH group. Age- and sex-matched healthy individuals were collected as a control group. A serum versus exosomes cohort (20 patients with PCa, 20 patients with BPH, and 20 control volunteers) (Table 1) and a follow-up exosomes cohort (51 patients with PCa and 40 control volunteers) (Table 2) were included in the present study. Clinical and biochemical features such as age, serum PSA, Gleason score, clinical stage, and metastasis of the patients were recorded in detail. PCa diagnosis was confirmed by histological examination or needle biopsy detected. Histopathologic Gleason score was performed based on the criteria of the World Health Organization, and classified as well differentiated (Gleason score ≤ 6), moderately differentiated (Gleason score = 7), and poorly differentiated/undifferentiated (Gleason score ≥ 8). Meanwhile, tumors were staged according to the seventh edition (2010) of the American Joint Committee on Cancer – Cancer Staging Manual. T-classification of PCa was divided as organ-confined (T1/T2) or nonorgan-confined (T3/T4).³¹

Table 1 Clinical characteristics of participants (serum vs exosomes cohort)

| Characteristic | PCa patients | BPH patients | Healthy controls | P-value |
|---------------------------|---------------------|---------------------|---------------------|-----------|
| Total number | 20 | 20 | 20 | |
| Age (years), median (IQR) | 69.50 (63.25–78.75) | 70.00 (64.25–75.75) | 72.50 (66.25–75.00) | 0.8029 |
| PSA (ng/mL), median (IQR) | 25.83 (7.29–189.50) | 7.64 (2.53–10.64) | 1.11 (0.71–2.31) | <0.0001** |
| Gleason score | | | | |
| ≤ 6 | 4 | | | |
| 7 | 10 | | | |
| ≥ 8 | 6 | | | |
| Clinical stage | | | | |
| T1/T2 | 15 | | | |
| T3/T4 | 5 | | | |
| Metastasis | | | | |
| No | 14 | | | |
| Yes | 6 | | | |

Note: ** $P < 0.01$.

Abbreviations: BPH, benign prostate hyperplasia; IQR, interquartile range; PCa, prostate cancer; PSA, prostate-specific antigen.

Table 2 Clinical characteristics of participants (follow-up exosomes cohort)

| Characteristic | PCa patients | Healthy controls | P-value |
|---------------------------|----------------------|---------------------|-----------|
| Total number | 51 | 40 | |
| Age (years), median (IQR) | 70.00 (64.00–76.00) | 70.50 (65.00–74.75) | 0.6919 |
| PSA (ng/mL), median (IQR) | 57.01 (14.33–196.00) | 1.03 (0.70–1.36) | <0.0001** |
| Gleason score | | | |
| ≤6 | 8 | | |
| 7 | 23 | | |
| ≥8 | 20 | | |
| Clinical stage | | | |
| T1/T2 | 24 | | |
| T3/T4 | 27 | | |
| Metastasis | | | |
| No | 31 | | |
| Yes | 20 | | |

Note: ** $P < 0.01$.

Abbreviations: IQR, interquartile range; PCa, prostate cancer; PSA, prostate-specific antigen.

Blood sampling and examination were performed prior to treatment, including surgery, radiotherapy, or chemotherapy. The peripheral blood was collected into serum-separator tubes and allowed to stand for 1 hour at room temperature, prior to centrifugation at $3,000\times g$ for 10 minutes. The resulting serum was transferred into fresh tubes and stored at -80°C for further analysis. The present study was approved by the Medical Ethics Committee of Xijing Hospital of Fourth Military Medical University, and written informed consent was obtained from all participants (No XJYYLL-2015129).

Exosome isolation

Exosomes were extracted from serum samples using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA), according to the manufacturer's instructions. Briefly, the serum was thawed on ice and centrifuged at $3,000\times g$ for 15 minutes to remove cells and cell debris. The supernatant was filtered through a $0.22\text{ }\mu\text{m}$ pore polyvinylidene fluoride filter (EMD Millipore, Billerica, MA, USA). Subsequently, one-fourth volume of ExoQuick solution was added to the supernatants and the samples were incubated at 4°C for 30 minutes, followed by centrifugation at $1,500\times g$ for 30 minutes. The final pellets, containing exosomes, were collected for characterization and RNA isolations (Figure S1).

Transmission electron microscopy

The exosomal pellets were resuspended in $50\text{ }\mu\text{L}$ phosphate-buffered saline and a drop of the suspension was placed on a sheet of parafilm. A copper grid was floated on the drop for 2 minutes at room temperature and was subsequently touched onto a drop of 2% phosphotungstic acid and stained for 2 minutes. The grid was allowed to dry for several minutes and

was examined. The images were captured using a Tecnai G2 Spirit electron microscope (FEI Co., Hillsboro, OR, USA).

Western blot

The total proteins were extracted from the serum exosomal pellets using radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and quantified using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Tokyo, Japan). Equivalent amounts of protein ($25\text{ }\mu\text{g}$) from exosomes and exosome-depleted serum supernatant were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred by electroblotting onto nitrocellulose membranes (Life Technologies, Carlsbad, CA, USA). Subsequently, the membranes were blocked for 1 hour at room temperature with blocking buffer (LI-COR Biosciences, Lincoln, NE, USA), and incubated overnight at 4°C with rabbit antihuman heat shock protein 70 antibody and CD63 antibody (1:1,000; RayBiotech, Norcross, GA, USA). β -Actin (1:1,000; Abcam, Cambridge, MA, USA) was used as loading control. After washing three times in Tris-buffered saline–Tween, the membranes were then incubated for 1 hour at room temperature with conjugated goat antirabbit immunoglobulin G (1:5,000; LI-COR Biosciences). The immunoreactive bands were visualized using an Odyssey Imaging system (LI-COR Biosciences).

Fluorescent-activated cell sorting

Fluorescent-activated cell sorting analysis of the serum exosomes was performed, as described previously.³² Briefly, the exosomes isolated from the serum were conjugated with $4\text{ }\mu\text{m}$ aldehyde/sulfate latex beads (Invitrogen Life Technologies),

followed by staining with phycoerythrin mouse antihuman CD63 or isotype control (BD Biosciences, San Jose, CA, USA). The analysis was performed using a BD FACScan flow cytometer (BD Biosciences).

Quantitative reverse transcription-polymerase chain reaction

Exosomal pelleted fraction isolated from 400 μ L serum was resuspended in 400 μ L nuclease-free water. Then, the total RNA containing miRNA was extracted from the exosomal pelleted suspensions or 400 μ L whole serum using an miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) with the final elution volume of 15 μ L. In the RNA isolation step, a total of 2 μ L synthetic *Caenorhabditis elegans cel-miR-39* (RiboBio, Guangzhou, the People's Republic of China) was added to each sample as a spike-in control.^{27,30} Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the PrimeScript RT Reagent kit and SYBR Premix Ex Taq kit (Takara Bio, Inc., Shiga, Japan). The reverse transcription reaction was carried out in 10 μ L containing 2 μ L of 5 \times PrimeScript buffer, 0.5 μ L of PrimeScript RT enzyme mix, 0.5 μ L of gene-specific primer, and 7 μ L of RNA extract. Reaction mixtures were incubated at 37°C for 15 minutes, 85°C for 5 seconds and then held at 4°C. Next, 2 μ L of complementary DNA was amplified with 10 μ L of 2 \times SYBR Premix Ex Taq, 0.8 μ L of gene-specific primers, 0.4 μ L of 50 \times ROX reference dye II, and 6.8 μ L of nuclease-free water in a final volume of 20 μ L. The qRT-PCR was run on an ABI 7500 Fast Detection system (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated at 95°C for 30 seconds, followed by 40 cycles at 95°C for 3 seconds and at 60°C for 30 seconds. At the end of the PCR cycles, melting curve analyses were performed to confirm the specificity of the PCR products. All the reactions were performed in triplicate. The bulge-loop qRT-PCR primers for *miR-141* and *cel-miR-39* were purchased from RiboBio. The relative expression levels of *miR-141* were normalized against *cel-miR-39* using the comparative $2^{-\Delta\Delta C_t}$ method.³³

Statistical analysis

The *miR-141* levels in the serum and the serum exosomal samples obtained from the same patients were compared using the Wilcoxon rank sum test. The Mann–Whitney *U*-test or Kruskal–Wallis test was used to determine the statistical significance of the *miR-141* levels in different samples. Correlation analysis was performed using the Spearman test. The area under the curve (AUC) was calculated through a receiver-operating characteristic (ROC)

curve analysis to determine the sensitivity and specificity of exosomal *miR-141* as a diagnostic biomarker for metastatic PCa. Statistical analyses were performed using SPSS statistical software package (version 17.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Exosomes are successfully isolated from human serum samples

To confirm the quality of the serum exosomes isolated using ExoQuick, the serum exosomes were characterized by morphological and protein marker analyses. Transmission electron microscopy analysis revealed that the microvesicle clusters isolated from serum were round or oval membrane vesicles with the size predominantly between 30 and 100 nm, and were homogeneous in appearance (Figure 1A). Western blot revealed that the specific exosomal protein markers, heat shock protein 70 and CD63, were expressed in isolated serum exosomal pellets as specific bands, but not in exosome-depleted serum supernatant (Figure 1B). Flow cytometric analysis also indicated that the isolated microvesicle clusters expressed CD63 (Figure 1C). These results demonstrated that the isolated serum particles exhibited characteristics of exosomes and confirmed the successful isolation of exosomes from human serum samples.

Expression of *miR-141* is significantly higher in exosomes compared with in serum

The expression of *miR-141* was determined in whole serum samples and serum exosomal pellets isolated from the same serum in a cohort of 20 patients with PCa, 20 patients with BPH, and 20 healthy individuals (Table 1). The qRT-PCR results revealed that, in the three groups, the expression levels of exosomal *miR-141* were all significantly higher compared with that in the serum circulating *miR-141* (control group, $P=0.0003$; BPH group, $P=0.0016$; PCa group, $P<0.0001$; Figure 2A). Furthermore, there were significant associations in the expression levels of *miR-141* between the whole serum and the exosomes (control group, $r=0.7474$ and $P=0.0002$; BPH group, $r=0.7368$ and $P=0.0002$; PCa group, $r=0.6692$ and $P=0.0013$).

In addition, the expression level of exosomal *miR-141* was compared among the three groups. The results indicated that the expression of exosomal *miR-141* in patients with PCa was significantly higher compared with that of the patients with BPH and the healthy controls (3.85-fold, $P=0.0007$ and 4.06-fold, $P=0.0005$, respectively; Figure 2B). Similar results were observed in the whole serum samples; however, this

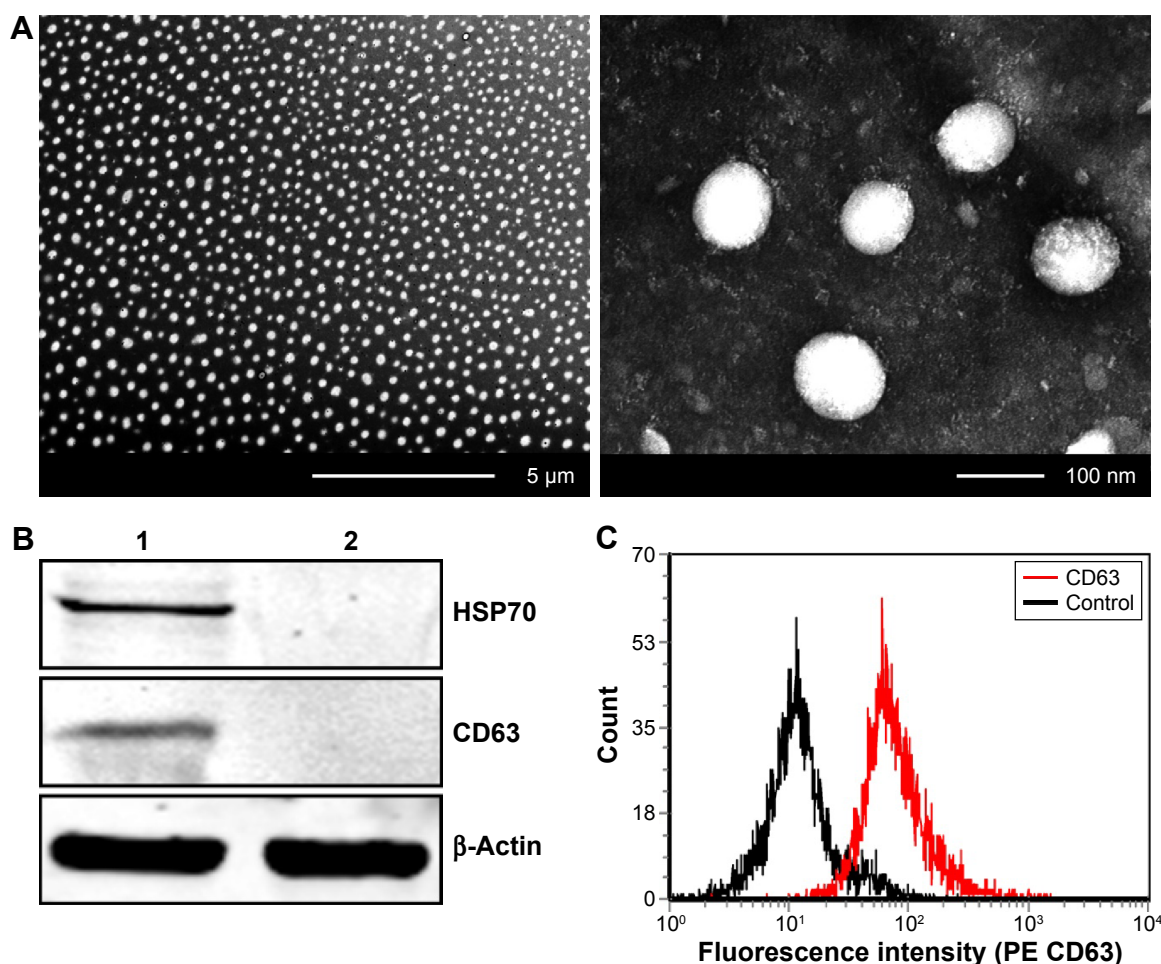


Figure 1 Characterization of isolated serum exosomes.

Notes: (A) Exosomes isolated from serum samples were homogeneous in appearance under transmission electron microscopy (scale bars, 5 μ m in left panel and 100 nm in right panel). (B) The expression levels of HSP70 and CD63 were detected in exosomes isolated from serum samples by Western blot analysis (lane 1, serum exosomal pellets; lane 2, exosomes-depleted serum supernatant). β -Actin was used as loading control. (C) The expression of CD63 was detected in exosomes isolated from serum samples by flow cytometry analysis.

Abbreviations: HSP70, heat shock protein 70; PE, phycoerythrin.

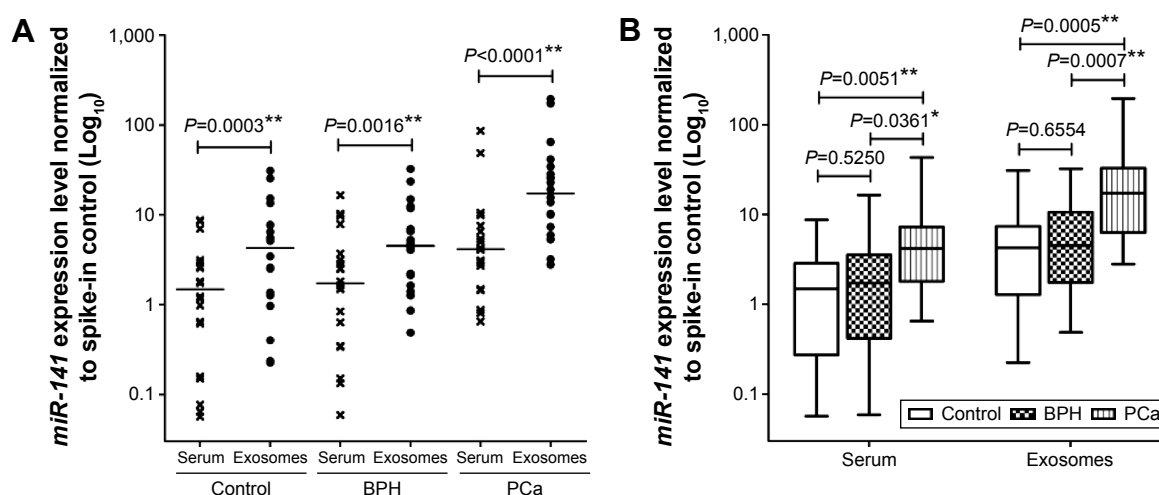


Figure 2 Expression of *miR-141* is significantly higher in exosomes compared with in serum.

Notes: The expression levels of *miR-141* in the serum and in the serum exosomes were measured in 20 patients with PCa, 20 patients with BPH, and 20 healthy volunteers. The expression levels of exosomal *miR-141* were analyzed by quantitative reverse transcription-polymerase chain reaction and normalized to a spike-in control (y-axis, Log_{10}). (A) The lines indicate the medians. A Wilcoxon rank sum test was used to determine the statistical significance. (B) The boxes represent the interquartile range. The horizontal lines in the boxes indicate the median levels. Whiskers indicate maximum and minimum values. A Mann-Whitney *U*-test or Kruskal-Wallis test was used to determine the statistical significance. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: BPH, benign prostate hyperplasia; miR, microRNA; PCa, prostate cancer.

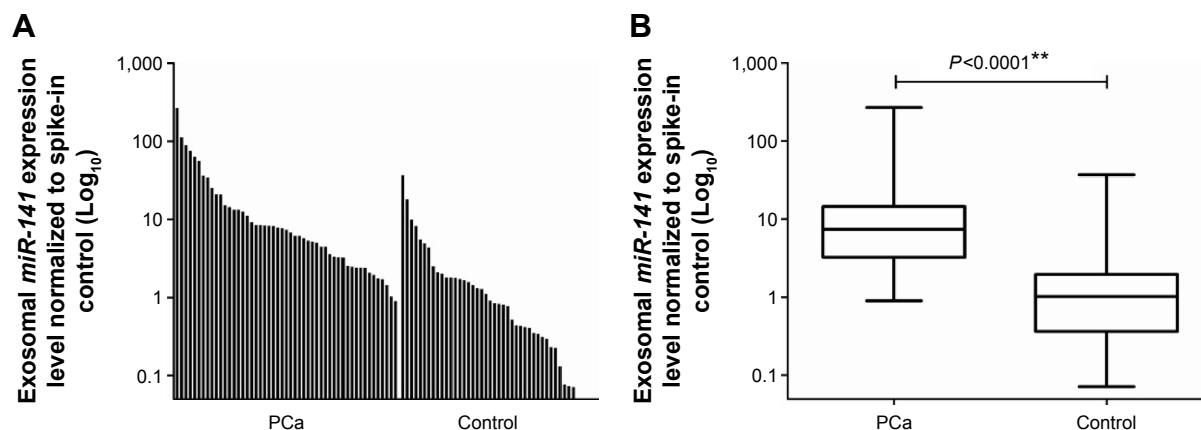


Figure 3 Expression of serum exosomal *miR-141* is significantly higher in patients with PCa.

Notes: (A and B) The expression levels of exosomal *miR-141* are illustrated in serum samples from 51 patients with PCa and 40 healthy individuals. The exosomal *miR-141* expression levels were analyzed by quantitative reverse transcription-polymerase chain reaction and were normalized against the spike-in control (y-axis, Log_{10}). (B) The boxes represent the interquartile range. The horizontal lines in the boxes indicate the median levels. Whiskers indicate maximum and minimum values. A Mann-Whitney U-test was used to determine the statistical significance. $^{**}P < 0.01$.

Abbreviations: miR, microRNA; PCa, prostate cancer.

was to a lesser extent (2.41-fold, $P=0.0361$ and 2.81-fold, $P=0.0051$, respectively; Figure 2B). The sensitivity of the circulating *miR-141* detection may be improved using exosomes.

Patients with PCa exhibit higher *miR-141* levels in serum exosomes

In order to understand the potential values of serum exosomal *miR-141* in the development and progression of PCa, the exosomal expression levels of *miR-141* in the 51 patients with PCa and the 40 healthy individuals in the follow-up cohort were determined using qRT-PCR (Table 2). As expected, the expression levels of exosomal *miR-141* in the PCa group were significantly increased compared with the control group ($P < 0.0001$; Figure 3A and B).

Exosomal *miR-141* levels correlate with the clinicopathological factors of patients with PCa

The 51 patients with PCa were divided into groups of high and low exosomal expression levels of *miR-141* using the median *miR-141* expression value as the cutoff point. In the high group, 42.31% of patients were aged ≥ 70 years, and in the low group, 60% of patients were aged ≥ 70 years ($P=0.085$). By contrast, the percentage of patients with PSA values ≥ 10 ng/mL was higher in the high group compared with the low group (92.31% vs 64.00%, $P=0.024$). The percentage of patients with a Gleason score ≥ 8 tumors and patients with T3/T4 diseases was higher in the high group compared with the low group (61.54% vs 16.00%, $P < 0.0001$; 73.08% vs 32.00%, $P=0.0026$). The majority of patients exhibited metastatic PCa in the high group compared with

the low group (69.23% vs 8.00%, $P < 0.0001$; Table 3). These results revealed that a higher PSA value, higher Gleason score, advanced T-classification, and metastasis were associated with higher serum exosomal expression of *miR-141* in patients with PCa.

Exosomal *miR-141* has potential value as a diagnostic biomarker for metastatic PCa

The present study, therefore, further focused on the association between serum exosomal expression of *miR-141*

Table 3 Association of exosomal *miR-141* expression with the clinicopathological characteristics of patients with PCa

| Characteristic | Total number | Exosomal <i>miR-141</i> | | P-value |
|----------------|--------------|-------------------------|-----------------------|-----------|
| | | Low expression group | High expression group | |
| Total number | 51 | 25 | 26 | |
| Age (years) | | | | 0.085 |
| <70 | 25 | 10 | 15 | |
| ≥ 70 | 26 | 15 | 11 | |
| PSA (ng/mL) | | | | 0.024* |
| <10 | 11 | 9 | 2 | |
| ≥ 10 | 40 | 16 | 24 | |
| Gleason score | | | | <0.0001** |
| <8 | 31 | 21 | 10 | |
| ≥ 8 | 20 | 4 | 16 | |
| Clinical stage | | | | 0.0026** |
| T1/T2 | 24 | 17 | 7 | |
| T3/T4 | 27 | 8 | 19 | |
| Metastasis | | | | <0.0001** |
| No | 31 | 23 | 8 | |
| Yes | 20 | 2 | 18 | |

Notes: * $P < 0.05$, ** $P < 0.01$.

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

and the tumor stage, metastasis, as well as on the potential value of exosomal *miR-141* as a diagnostic biomarker for PCa.

First, serum exosomal expression of *miR-141* in the patients with different Gleason scores was determined. As shown in Figure 4A, significant differences were observed when patients with a Gleason score ≥ 7 tumors were compared with the controls ($P < 0.0001$). However, no statistically significant difference was observed between low Gleason score (≤ 6) tumors and the controls ($P = 0.0619$).

Subsequently, the levels of serum exosomal *miR-141* in the 20 patients with metastatic PCa were compared with the 31 patients with localized PCa. The median exosomal *miR-141* levels were 21.019 in the metastatic group and 4.449 in the localized group ($P < 0.0001$). Each group was significantly higher compared with the control group ($P < 0.0001$; Figure 4B).

Subsequently, ROC curves were generated in order to assess the power of exosomal *miR-141* to distinguish the patients with metastatic PCa from the patients with

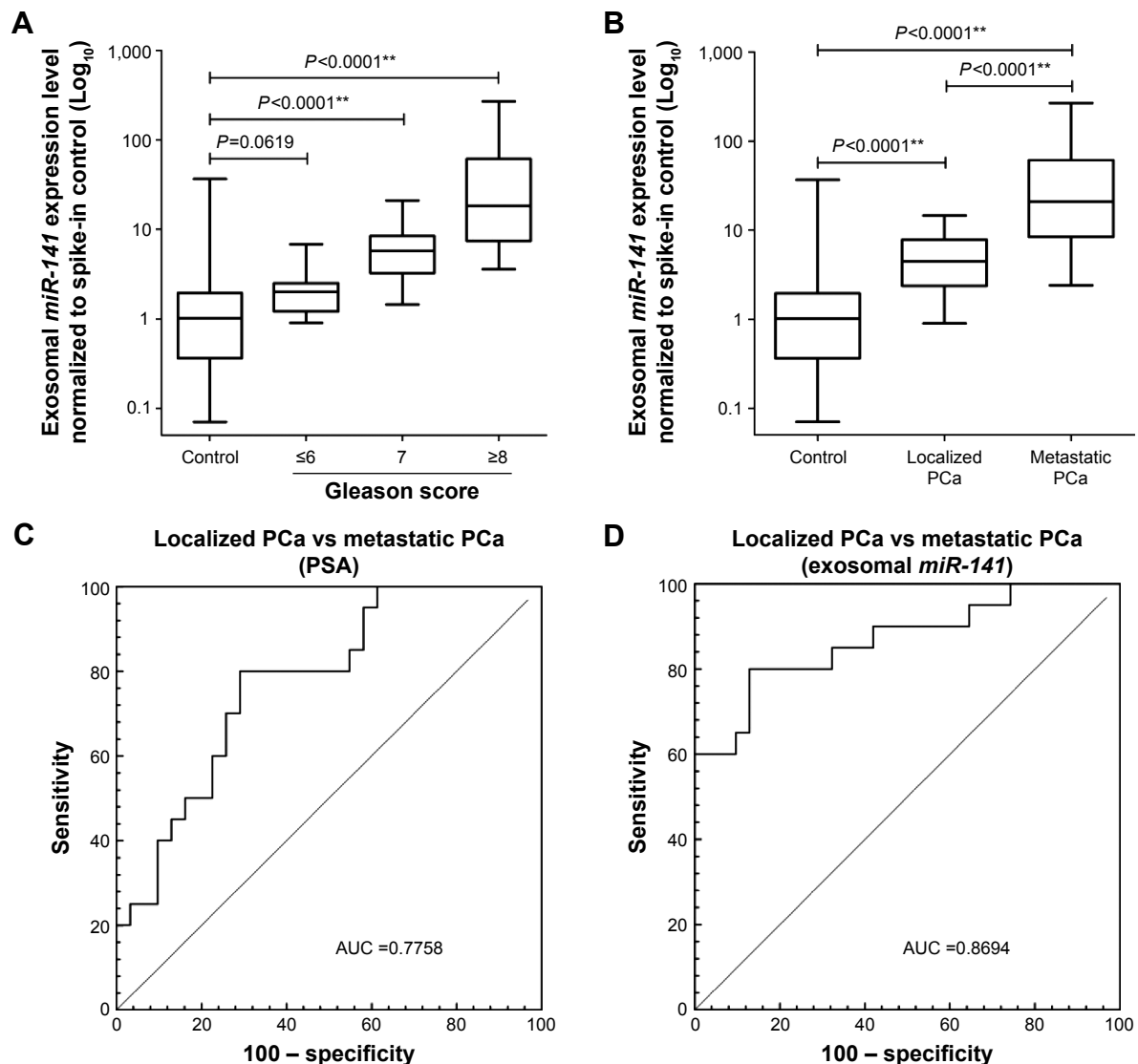


Figure 4 Expression levels of serum exosomal *miR-141* in subgroups of patients with PCa.

Notes: (A) A box plot of serum exosomal *miR-141* levels across the PCa Gleason score. Quantitative reverse transcription-polymerase chain reaction was performed and normalized against the spike-in control (y-axis, Log₁₀). The boxes represent the interquartile range. The horizontal lines in the boxes indicate the median levels. Whiskers indicate maximum and minimum values. A Mann–Whitney *U*-test or Kruskal–Wallis test was used to determine the statistical significance. (B) The expression levels of serum exosomal *miR-141* were measured in 20 patients with metastatic PCa, 31 patients with localized PCa, and 40 healthy individuals by quantitative reverse transcription-polymerase chain reaction and were normalized against the spike-in control (y-axis, Log₁₀). The boxes represent the interquartile range. The horizontal lines in the boxes indicate the median levels. Whiskers indicate maximum and minimum values. A Mann–Whitney *U*-test or Kruskal–Wallis test was used to determine the statistical significance. Receiver-operating characteristic analysis of (C) PSA and (D) exosomal *miR-141* was used to differentiate between patients with metastatic and patients with localized PCa. PSA yielded an AUC of 0.7758 with 80% sensitivity and 70.97% specificity in discriminating metastatic PCa. Exosomal *miR-141* yielded an AUC of 0.8694 with 80% sensitivity and 87.10% specificity in discriminating metastatic PCa. $^{**}P < 0.01$.

Abbreviations: AUC, area under the curve; miR, microRNA; PCa, prostate cancer; PSA, prostate-specific antigen.

localized PCa. The analyses revealed that the exosomal *miR-141* had a higher AUC (AUC=0.8694, 95% confidence interval =0.7625–0.9762) compared with the PSA (AUC =0.7758, 95% confidence interval =0.6486–0.9030). At the cutoff value of 8.362 for the relative expression of exosomal *miR-141*, the sensitivity was 80% and the specificity was 87.10%. At the cutoff value of 60.13 for PSA, the sensitivity was 80% and the specificity was 70.97% (Figure 4C and D). The ROC curve indicated that exosomal *miR-141* was an improved discriminator to distinguish between patients with metastatic PCa and those with localized PCa.

Discussion

The RNA sequencing analysis of plasma-derived exosomes revealed that miRNAs were the most abundant exosomal RNA species, comprising 76.20% of all mappable reads.³⁴ In addition, Gallo et al¹³ indicated that the majority of circulating miRNAs that were detectable in the serum and saliva were concentrated in exosomes and that exosome isolation may improve the sensitivity of miRNA amplification from human biological fluids. A previous study reported that exosomes provide a protective and enriched source of miRNA for biomarker profiling, and the exosomes isolated from serum contained a higher percentage of miRNA compared with the plasma.³⁵ In addition, it was demonstrated that serum exosomes may serve as a suitable material to measure circulating miRNA biomarkers for the diagnosis and prognosis of diseases.^{36,37} A previous study reported that a set of circulating exosomal miRNAs can be used as the noninvasive diagnostic biomarker of colon cancer.³⁸ Tanaka et al⁷ confirmed that serum expression of exosomal miR-21 expression was upregulated in patients with esophageal squamous cell carcinoma and was positively correlated with tumor progression and aggressiveness. Taken together, these findings suggest that the serum exosomal miRNAs may be important in noninvasive diagnostic testing. Therefore, the aim of the present study was to determine if exosomes derived from patients with PCa were valuable for cancer diagnosis.

It has been previously reported that the expression levels of *miR-141* are correlated with a high Gleason score or metastasis of the patients with PCa.^{14,27–30} However, the comparison of the expression levels of *miR-141* in the serum samples and exosomes isolated from the serum of patients with PCa remains to be elucidated. In order to determine whether exosomes are an improved material in comparison with the total serum for miRNA testing, *miR-141* was used as an example to compare the miRNA expression levels in the whole serum and the serum exosomes of patients with

PCa, BPH, and healthy volunteers. Furthermore, the potential clinical significance of exosomal *miR-141* for the detection of PCa was also assessed.

First, the expression levels of *miR-141* were analyzed by qRT-PCR in the whole serum samples and serum exosomal pellets isolated from the same serum in a cohort of 20 patients with PCa, 20 patients with BPH, and 20 healthy control individuals. This revealed that the expression of exosomal *miR-141* in patients with PCa was significantly higher compared with the patients with BPH and the healthy controls. In addition, significant correlations were observed in the *miR-141* expression levels between the whole serum and exosomes, and the expression levels of exosomal *miR-141* were all significantly higher compared with the serum circulating *miR-141* in the three groups. Therefore, it was suggested that *miR-141* was enriched in exosomes, which provides increased sensitivity of detection compared with the whole serum.

In the second validation set, significantly elevated levels of serum exosomal *miR-141* were observed in patients with PCa, and were considerably correlated with cancer metastasis. However, no significant difference was observed between a low Gleason score tumor and the controls. It was suggested that serum exosomal *miR-141* failed to facilitate early detection of the primary PCa.

However, the ROC curve indicated that, in comparison with PSA, exosomal *miR-141* was an improved discriminator to distinguish between patients with metastatic PCa and those with localized PCa. Therefore, it is likely that the serum exosomal *miR-141* was more associated with metastatic PCa disease compared with the grading of primary PCa. These observations suggested that serum exosomal miRNAs, including exosomal *miR-141*, may be useful to predict the risk of metastatic PCa, which is consistent with previous reports regarding circulating *miR-141*.

However, the role of exosomal *miR-141* in PCa progression remains to be elucidated and must be further examined in functional studies. The exact role of *miR-141* in cancer requires further investigation, particularly in metastatic PCa.

In conclusion, the present study demonstrated that serum exosomes were a more accurate material compared with the whole serum for measuring circulating *miR-141* levels in patients with PCa. Exosomal *miR-141* may be used as a valuable noninvasive biomarker for the detection of metastatic PCa. To the best of our knowledge, the present study compared the differences between miRNA analyses using total serum and the serum exosomes for the first time, and

evaluated the potential clinical significance of serum exosomal miR-141 for the detection of metastatic PCa.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

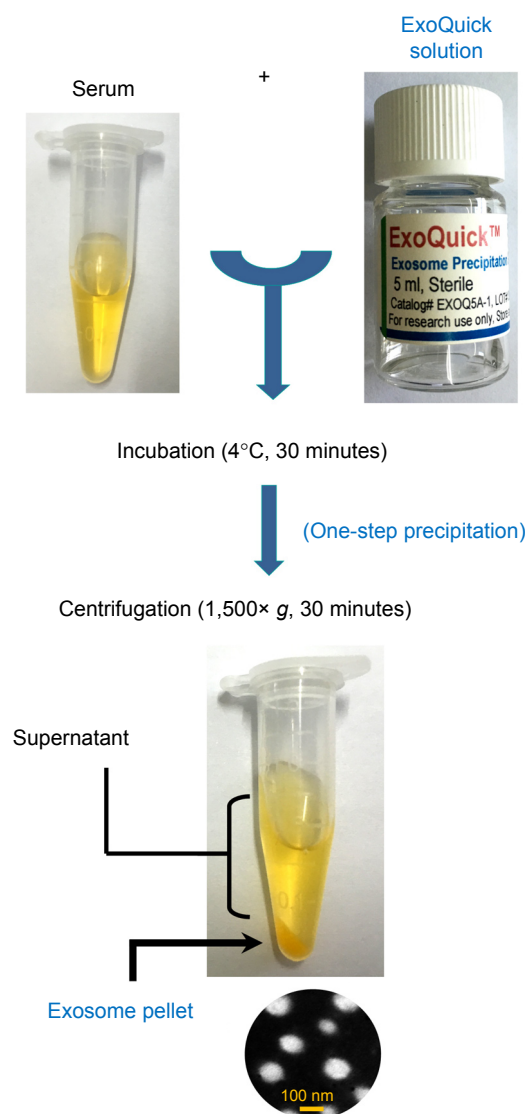


Figure S1 Flow chart for exosomal isolation.

Notes: Exosomes were extracted from serum sample using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA). A quarter volume of ExoQuick solution was added to serum and the samples were incubated at 4°C for 30 minutes, followed by centrifugation at 1,500× g for 30 minutes. The final pellets, containing exosomes, were collected for characterization and RNA isolation.

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