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Plasma exosomal miR-125a-5p and miR-141-5p as non-invasive biomarkers for prostate cancer

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Predictive biomarkers for early diagnosis of prostate cancer are important for its treatment. The functional microRNAs in the exosomes of plasma and serum samples are of interest as stable and non-invasive biomarkers for recurrence in cancer patients. The present study aimed to clarify the value of plasma exosomal miRNA-125a-5p and miR-141-5p as biomarkers for the diagnosis of prostate cancer. The study included 19 healthy individuals and 31 prostate cancer patients. In comparison to the levels in healthy controls, exosomal miR-141-5p levels showed a slight increase in prostate cancer patients (p=0.085), and miR-125a-5p levels that showed a significant decrease in patients with prostate cancer than in healthy controls (p=0.032). As a derived parameter, the miR-125a-5p/miR-141-5p ratio was significantly higher in patients with prostate cancer than in healthy controls (p<0.001). We found that exosomal miR-141-5p in plasma showed a promise in distinguishing prostate cancer patients with the AUC of 0.652, and for miR-125a-5p, the AUC was 0.691. For the miR-125a-5p/miR-141-5p ratio, the AUC value was 0.793. We found that miR-125a-5p has a weak positive correlation with PSA (correlation coefficient = 0.3413). Moreover, miR-141-5p has been found to hold a negatively no-significant correlation with PSA, with the correlation coefficient is -0.1102. We speculate that, as diagnostic markers for prostate cancer, miR-125-5p and miR-141-5p might be independent of the PSA. In summary, the results of this study suggest that high plasma exosomal expression of miR-141-3p and low expression of miR-125a-5p in plasma exosomes from prostate cancer patients might be useful markers of specific tumor traits associated with prostate cancer. Moreover, the miR-125a-5p/miR-141-5p ratio seems to perform better than either of the single values alone.

Key words: prostate cancer, miR-125a-5p, miR-141-5p, biomarkers

Prostate cancer is one of the most common malignant tumors in male patients, and its incidence is the second most frequent in male patients [1]. Because the early symptoms are not significant, most of the patients are in the late stage when they see a doctor. Therefore, early diagnosis and early treatment are of great significance to the prognosis of patients. Exosomes are extracellular vesicles (EVs), which are secreted by a variety of cells, and contain a large number of proteins, lipids, and nucleic acids [2]. The most important function of exosomes is to participate in intercellular communication. Exosomes act on target cells in different ways to realize the transmission of information between cells and then produce a series of physiological or pathological reactions, such as angiogenesis, immune regulation, and antigen [3].

Studies have indicated that miRNAs are stably present in the exosomes of many cancer patients, suggesting that exosomal miRNAs can be explored as biomarkers for cancer diagnosis and prognosis [4]. It is reported that LNcaP, a prostate cancer cell line, has increased exosomal release under conditions of growth factor HB-EGF stimulation or DIAPH3 silencing, and the inclusion of miR-125a reduces the expression of AKT1 in macrophages and monocytes and promotes tumor cell proliferation [5]. miR-125a-5p has also been shown to have decreased expression in breast cancer [6], and upregulation of miR-125a-5p induces apoptosis in lung cancer [7]. Plasma exosomal miR-125a has also been reported as a diagnostic biomarker for early-stage colon cancer [8]. Alexandre's research shows that miR-145 is involved in the MYC and RAS pathway and can be supposed as a potential molecule for the treatment of metastatic prostate cancer patients [9]. Furthermore, miR-145 in urinary EVs (UEVs) was upregulated in prostate cancer patients, and the miR-145

of UEVs is recommended as a biomarker for prostate cancer patients [10].

In this study, we investigated associations between circulating exosomal miR-125a-5p and miR-141-5p at the time of diagnosis (before receiving any treatment) in prostate cancer patients. Healthy control cases were also enrolled. The study aimed to evaluate whether the exosomal miR-125a, miR-141-5p, and miR-125a/miR-141-5p ratio would potentially serve as useful biomarkers for prostate cancer diagnosis.

Patients and methods

Patient characteristics. This study included 19 healthy individuals (age distribution ranged from 46 to 78 years) and 31 prostate cancer patients (age distribution ranged from 52 to 88 years, clinical stages I/II, n=19; III/IV, n=12), recruited during 2018 and 2019 from the 928th Hospital of the PLA Joint Logistics Support Force. Prostate cancer patients were required to provide medical history with pathological histological diagnosis. The control group was composed of healthy honors without a cancer history. Routine clinical blood samples were collected at the time of diagnosis and were analyzed by the hospital laboratory according to customs procedures. Blood was collected from each subject, centrifuged at 800×g for 15 min at 4 °C, and the isolated plasma samples were stored at –80 °C until exosome isolation for qPCR analysis. Electrochemiluminescence immunoassay (Abbott i1000 chemiluminescence immunoassay, and its matching reagent) was used for PSA detection in venous blood.

Exosome isolation and characterization. Exosomes were isolated from 1000 µl plasma using the exoEasy Maxi Kit (QIAGEN, Catalog #76064, Germantown, MD, USA) according to the protocol. Additionally, three random samples were selected for the characterization of the isolated vesicles. Exosome particle size and concentration were measured using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany). For each sample, 1 ml of the sample was diluted using 1× PBS buffer, and the nanoparticle tracking analysis (NTA) measurement was recorded and analyzed at different positions. The mean, median, and the concentration of the sample were calculated subsequently. For transmission electron microscopy (TEM), 30 µl of the exosomal sample was fixed to a mesh grid for 2 min, 2% aqueous solution of uranyl acetate was fixed for 5 min, and the sample was observed under a transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

Exosomal RNA extraction and quantitative reverse transcription-polymerase chain reaction. Exosomes were isolated using the exoEasy Maxi Kit (QIAGEN, Catalog Number76064, Germantown, MD, USA) according to the manufacturer's instructions. Total RNA was extracted from exosomes using a miRNeasy kit (QIAGEN) according to the manufacturer's instructions. The RNA was reverse transcribed using a QIAGEN miRNA Reverse Transcription Kit (QIAGEN, Germantown, MD, USA). Following cDNA synthesis, the levels of miR-141-5p, miR-125-5p, and hsa-miR-16-5p (as a reference) were determined in triplicate and analyzed using a real-time PCR detection system (LineGene K Plus, Hangzhou Bioer Technology Co. Ltd.) and SYBR green qPCR master mix (QIAGEN) according to the manufacturer's instructions. The primers of hsa-miR-125a-5p:5'-ACACTCCAGCTGGGTCCCTGAGACCCTTTAAC-3'(forward), 5'-TGGTGTCGTGGAGTCG-3'(reverse), the primers of hsa-miR-141-5p: 5'-CCCTGTAGCAACTGGT-GAGC-3'(forward), 5'-CCCTGAAGGTTACTGCCGAG-3'(reverse), the primers of hsa-miR-16-5p: 5'-TAGCAG-CACGTAAATATTGGCG-3'(forward), 5'-TGCGTGTCGT-GGAGTC-3'(reverse). The PCR reactions were run using a 7900 HT Fast Real-Time PCR system (Applied Biosystem, Foster City, USA). Data normalization was performed using normalizer assay CT, Ct=dCt. Melting curve analyses were performed to confirm the specificity of the PCR products. The expression of miRNA was normalized (ΔCt) to the hsa-miR-16-5p, where ΔCt=Ct(miR-141-5p, miRNA 125-5p) – Ct(hsa-miR-16-5p).

Statistical methods. Statistical analysis was performed with the R (V3.5.1) statistical language. The miRNA experiments were performed in triplicate, and the results are presented as the mean ± standard deviation. Correlations between continuous data were determined by Pearson product correlation analysis. The receiver operating characteristic (ROC) curve area was used to analyze the clinical diagnostic value of miR-125a-5p and miR-141-5p in the detection of early prostate cancer. A $p<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of isolated exosomes. Exosomes were isolated from plasma using the exoEasy Maxi Kit. To examine the size distribution and morphology of the isolated exosomes, the exosome pellets were examined by NTA and TEM. TEM indicated that the exosomes had a spherical shape with a diameter of approximately 40–180 nm. NTA analysis revealed that the size distribution of exosomes ranged from approximately 50 to 200 nm (Figure 1). The TEM and the NTA confirmed (based on their morphology and size) that the vesicles isolated from the conditioned media were exosomes.

Prognostic role of plasma exosomal miR-141-5p and miR-125a-5p in patients with prostate cancer. The expression levels of miR-141-5p and miR-125a-5p, as well as the miR-141-5p/miR-125a-5p ratio, were measured. We found that miR-141-5p was higher in patients with prostate cancer than in healthy controls, but without reaching statistical significance ($p=0.085$), whereas miR-125a-5p was significantly higher in patients with prostate cancer than in healthy controls ($p=0.032$). As a derived parameter, the miR-125a-5p/miR-141-5p ratio was significantly higher in patients

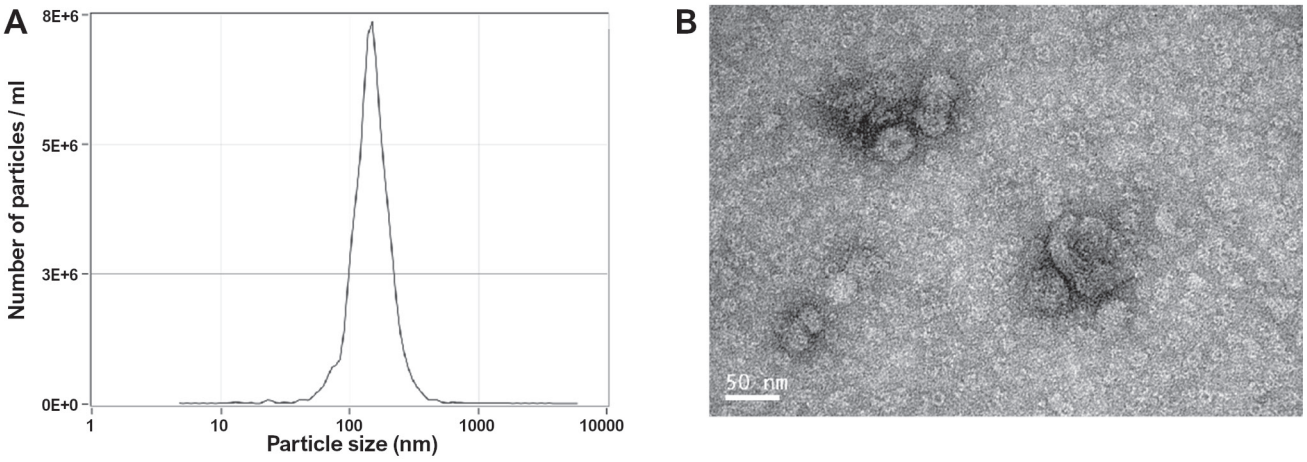

Figure 1. Characterization of exosomes derived from plasma samples. Size distribution of exosomes through NTA revealed that the size of exosomes ranged from approximately 50 to 200 nm (B). TEM showed that exosomes had a rounded morphology and ranged from 40 to 180 nm (B, scale bar = 50 nm).

with prostate cancer than in healthy controls (p<0.001, Figures 2A–C). Because the qPCR value was performed using dCT (normalizer assay CT), higher values indicate lower levels of expression. To assess the potential prognostic role of exosomal miRNA expression levels, miR-141-5p and miR-125a-5p were quantified in plasma from the subjects, which included healthy cases and prostate cancer patients. We used ROC curve analysis to evaluate the predictive value of exosomal miR-141-5p and miR-125a-5p. Overall, exosomal miR-141-5p in plasma showed potential in distinguishing prostate cancer patients, with an AUC of 0.652. For miR-125a-5p, the AUC was 0.691. For the miR-125a-5p/miR-141-5p ratio, the AUC was 0.793 (Figures 2D–F).

The correlation of plasma exosomal miR-141-5p, miR-125a-5p, and PSA. Prostate-specific antigen (PSA) is a protein produced by both cancerous and noncancerous tissue in the prostate, and the PSA test is used primarily to screen for prostate cancer. In this study, the correlations between plasma exosomal miR-141-5p, miR-125a-5p, and PSA were calculated. We found that the miR-141-5p has a weak negative correlation with PSA (R=–0.1102, p=0.446), and miR-125a-5p has a weak correlation with PSA (R=0.3413, p=0.015, Figure 3).

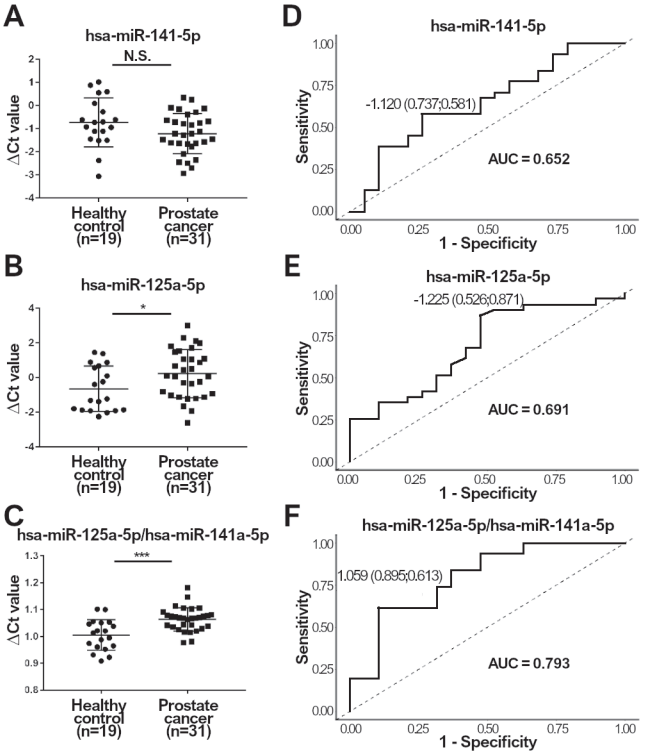

Figure 2. Prognostic role of plasma exosomal miR-141-5p and miR-125a-5p in patients with prostate cancer. Expression of miR-141-5p (A), miR-125a-5p (B), and their levels ratio (C) in patients with prostate cancer and in healthy controls are shown. D–F) ROC curve analysis of miR-141-5p, miR-125a-5p (B), and their levels ratio. *: p<0.050, ***: p<0.001, NS: not significant.

Discussion

It is well known that exosomes play an important role in tumor cells and are associated with tumor progression, metastasis, and drug resistance [11]. Exosomes carry many biomolecules, including miRNAs, which are shed from tumor cells and are cancer-specific. Exosomes have been found to be highly stable and a rich source of biomarkers in biological fluids. miRNAs are carried by exosomes, play an important role in a variety of cancers, and are used as biomarkers for tumor diagnosis and prognosis [12]. Measurement of miRNA in exosomes better reports the tumor environment compared to measurements made directly in serum or plasma. Studies have reported that prostate cancer-associated fibroblast-

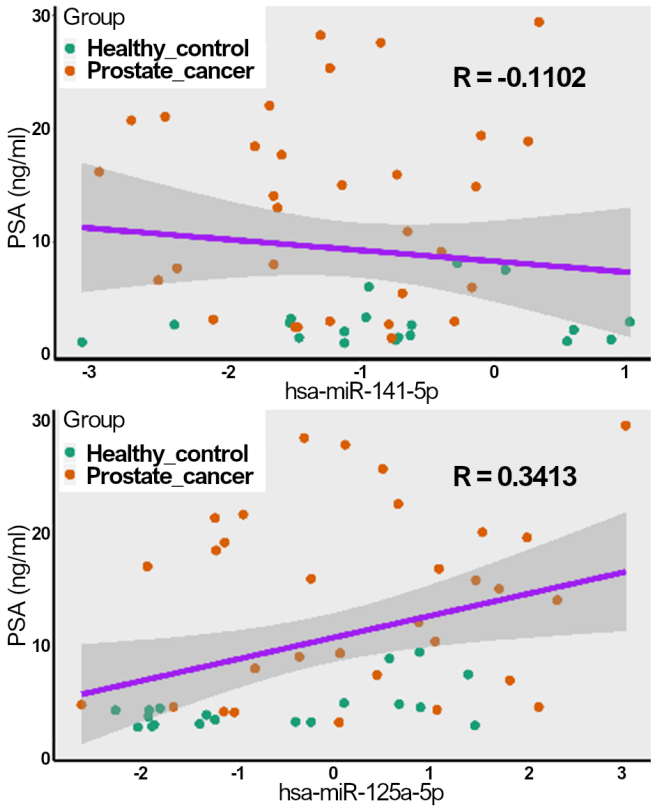

Figure 3. The correlations between exosome miR-141-5p, miR-125a-5p, and PSA. The miR-141-5p has a weak, non-significant, negative correlation with PSA (R=–0.1102, p=0.446) and miR-125a-5p has a weak correlation with PSA (R=0.3413, p=0.015).

derived exosomes can re-encode metabolic mechanisms after being ingested by prostate cancer cells, inhibit mitochondrial oxidative phosphorylation, and increase glycolysis and glutamine-dependent reduction [13]. Fibroblast-derived exosomes can carry amino acid, lipid, and tricarboxylic acid circulating intermediates, allowing prostate cancer cells to grow under nutrient-deficient conditions [14, 15].

Obtaining a stable circulating source is a prerequisite for exosomes research. There are many ways to isolate and detect exosomes. Physical separation of exosomes from cells and particles of similar size is challenging due to the complexity of body fluids. The use of differential ultracentrifugation to separate exosomes is a classic method but results in incomplete separation of proteins and other contaminants. Separation of extracellular vesicles by size-exclusion chromatography has been shown to provide higher centrifugation recovery, although size-based techniques alone cannot distinguish exosomes from other vesicle types. In this study, we choose the exoEasy Maxi Kit for purification of exosomes, which uses a membrane-based affinity binding step to separate exosomes and other EVs from serum, plasma, or cell culture supernatants.

In this study, we observed the expression of miR-125a-5p and miR-141-3p, largely because previous studies have reported that these have a relatively clear correlation with cancer. It is reported that the decrease of miR-125a-5p expression in gastric cancer is closely related to tumorigenesis and poor prognosis [16], and miR-125a-5p can inhibit the invasion and migration of hepatic cancer cells by regulating the activity of the PI3K/AKT/mTOR pathway [17]. On the other hand, high levels of miR-141-3p expression have been reported to target PTEN, which in turn leads to activation of the PI3K-Akt pathway [18]. Elevated levels of miR-141 are associated with the development of colorectal cancer and poor prognosis [19]. Exosomal miR-141-3p from prostate cancer cells was found to promote osteoblast activity and regulated the microenvironment of bone metastases and, subsequently, to promote bone metastasis of prostate cancer in *in vivo* and *in vitro* studies [20]. So, the ratio of miR-125a-5p/miR-141-5 can better reflect the active state of the PI3K/AKT/mTOR pathway, thereby indirectly predicting the possibility of tumor occurrence and development.

In this study, the expression levels of exosomal miR-141-5p and miR-125a-5p, as well as the miR-141-5p/miR-125a-5p ratio, were determined. We found that miR-141-5p has a rising trend, but without significant difference (p=0.085), and miR-125a-5p expression is lower in patients with prostate cancer than in healthy controls (p=0.032). As a derived parameter, the ratio of miR-125a-5p/miR-141-5p was significantly higher in patients with prostate cancer than in healthy controls (p<0.001).

PSA is produced by normal cells and malignant cells of the prostate gland. The blood levels of PSA are usually elevated in men with prostate cancer, and the PSA test was initially approved by the FDA to monitor prostate cancer progression in men who have been diagnosed with prostate cancer [21]. Patients with prostate symptoms always receive a PSA test to help the doctor make an accurate diagnosis [22]. In this study, we analyzed the correlation between miR-125a-5p, miR-141-5p, and PSA. We found that the ratio of miR-125a-5p/miR-141-5 had a weak positive correlation with PSA (correlation coefficient R=0.2251, data not shown). In this study, exosomal miR-141-5p and miR-125a-5p cannot increase the diagnostic accuracy of PSA. So, we speculate that, as a diagnostic marker for prostate cancer, miR-125-5p and miR-141-5p might be independent of PSA. However, due to the small number of patients enrolled in this study, this conclusion will need to be tested in future clinical studies using large sample sizes.

In conclusion, we have demonstrated a link between the expression of miR-125 and miR-141-3p in plasma exosomes and the presence of prostate cancer. We suggest that high levels of miR-141-3p and low levels of miR-125a-5p in plasma exosomes from prostate cancer patients might act as markers of specific tumor traits associated with prostate cancer. Also, the miR-125a-5p/miR-141-5p ratio seems to outperform either of these values alone.

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