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CD81 and CD117 Surface Markers Profiling of Prostate Cancer Urinary Exosomes Using CD9 Magnetic Beads

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Abstract Prostate cancer is one of leading cancers in men. While some types of prostate cancer grow slowly and may need minimal or no treatment, other types are aggressive and can spread quickly. The markers capable to better distinguish cancerous from benign conditions and slow-growing cancers from aggressive ones are required. Present study is aimed to analysis of expression of CD9, CD81, and CD117 membrane proteins on the exosomes isolated from the urine of patients with histologically confirmed prostate cancer and age-matched patients without cancer.

Keywords Urinary exosomes · Prostate cancer · CD9 · CD81 · CD117

1 Introduction

Prostate cancer (PCa) is one of leading cause of cancer-related deaths in man [1, 2]. Most of prostate cancers grow slowly and may need minimal or no treatment; however, some types are aggressive and can spread quickly. Thus, distinguishing cancerous from benign conditions as well as slow-growing cancers from aggressive ones is crucial for disease outcome. Prostate cancer is one of few cancers for which a clinical useful protein biomarker is determined for diagnostics and follow-up after treatment. Prostate-specific antigen (PSA) can detect early PC and predict response to treatment [3]; however, its specificity is low since benign hyperplasic conditions can also be associated with a PSA increase [4]. Therefore, additional biomarkers capable to predict the risk for future metastases are required. One of the markers recently proposed for characterization of cancer aggressivity is mast/stem cell growth factor receptor (SCFR), also known as CD117 [5]. It was shown that the number of circulating $CD117^+$ cell is increasing with cancer progression and correlated with high PSA values. Staining of CD117 and its ligand stem cell factor (SCF) are both upregulated with cancer severity, with the highest expression occurring in bone metastatic prostate cancer tumors. In a xenograft model, CD117-enriched tumors were more vascularized and aggressive. Therefore, CD117 overexpression could be a biomarker for prostate cancer diagnosis, prognosis, and/or response to therapy [5].

The new approach in modern medicine is the concept of "liquid biopsy," where the molecular tumor markers are analyzed in bio-fluid samples. Besides to circulated tumor cells and cell-free nucleic acids, extracellular vesicles (exosomes) are used in liquid biopsy tests. Exosomes are the population of small (40–100 nm) vesicles of endocytic origin. Exosomes carry RNAs and proteins and are generally recognized as agents of intercellular communication [6, 7]. They are found in natural body fluids—in blood, saliva, urine, and breast milk. Recently, it was demonstrated that exosomes participate in metastasis and cancer progression (for review see [8]).

Protein profiling of urinary exosomes is powerful non-invasive screening tool for many urinary tract disorders including prostate cancer. One of the approaches allowing accurate quantification of exosomal transmembrane proteins is application of magnetic beads coated with specific antibodies. The aim of present work was optimization of the experimental protocol allowing reliable bead-based quantification

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of CD81$^+$ and CD117$^+$ prostate cancer urinary exosomes using anti-CD9 magnetic beads. To our knowledge, this is the first attempt to characterize the expression of CD117 in urinary exosomes of prostate cancer patients.

2 Materials and Methods

Urine samples from 10 men with and without prostate cancer were collected after digital rectal exam (DRE) and kept at −80 °C. Urinary exosomes were isolated by differential centrifugation as described in [9]. Transmission electron microscopy (TEM) was used to analyze the purity of isolated exosomes. Quantification of CD9$^+$ exosomes was performed either with *Exosome-Human CD9 Flow Detection Reagent* (ThermoFisher Scientific). Surface marker profiling was performed by incubation of anti-CD9-coated magnetic beads either with pre-purified exosomes (5 ml of urine per sample) or directly with 5 ml of patient's urine for 36 h at 4 °C followed by incubation of exosome-bead complexes with CD81-PE or CD117-PE and flow analysis.

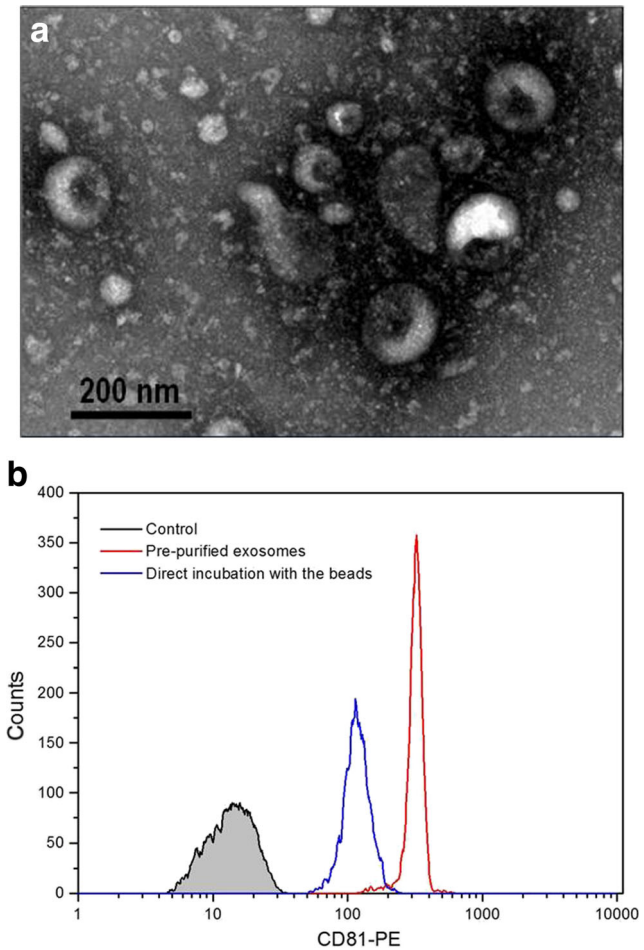

Fig. 1 **a**. TEM image of pre-purified exosomes. **b**. Comparison of exosomes analyzed directly or purified by differential centrifugation

Table 1 List of the patients with the PSA value and diagnoses

Patients	Total PSA, ng/ml	Gleason score	Histology
1	7.96	–	No atypical epithelium
2	5.31	–	No atypical epithelium
3	5.75	–	No atypical epithelium
4	5.96	5 (2 + 3)	Prostate cancer
5	5.14	6 (3 + 3)	Prostate cancer
6	10.33	6 (3 + 3)	Prostate cancer
7	12.32	6 (3 + 3)	Prostate cancer
8	14.30	6 (3 + 3)	Prostate cancer
9	24.11	7 (4 + 3)	Prostate cancer
10	11.64	8 (3 + 5)	Prostate cancer

3 Results and Discussion

TEM image of 100,000 g pellet shows the typical for exosomes morphology and vesicles size distribution (Fig. 1a). Since general exosomal markers (CD9, CD81, and CD63) are typically overrepresented as compared to organ-specific ones, the sensitivity of detection method is crucial for reliable detection of cancer markers. To analyze the impact of exosomes pre-enrichment to bead-based detection

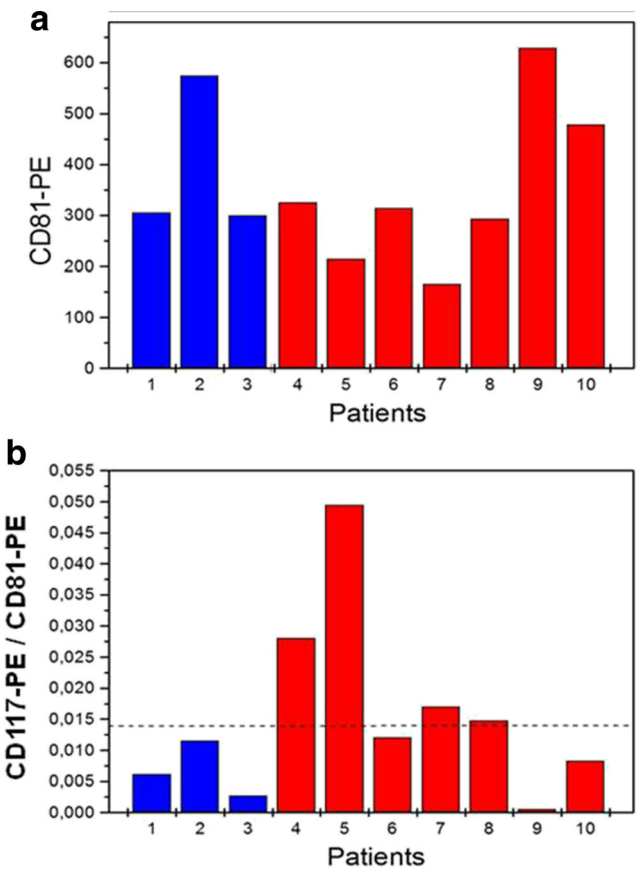

Fig. 2 CD81 (**a**) and CD117 (**b**) membrane proteins detection on CD9$^+$ urinary exosomes

sensitivity, anti-CD9-coated magnetic beads were either incubated with pre-purified exosomes or directly with urine of cancer patient followed by CD81 staining of exosome-bead complexes (Fig. 1b). Since the higher value of fluorescence signal was obtained with exosomes, isolated by differential centrifugation, the further experiments were made with pre-purified exosomes.

Expression of CD81 and CD117 protein markers was analyzed on $CD9^+$ exosomes purified from urine of three patients without PCa and seven patients with histologically confirmed prostate cancer of different grade (Table 1). The values of CD81-PE fluorescence for different patients are shown in Fig. 2a. Our data demonstrate essential difference in CD81 expression between the patients. No direct correlation between CD81 value and cancer grade was observed. Since tetraspanins CD9 and CD81 are believed to be general exosomal markers, such difference just reflects the variation in exosomes concentration in urine samples of different patients. Therefore, taking into account the absolute value of fluorescent signal corresponding to the marker of interest (CD117 in our study) could give wrong information concerning the portion of marker-positive exosomes. Hence, the normalization is required. We normalized CD117 signal value measured for each patient to the value of CD81 signal that represents the portion of $CD117^+$ exosomes in total fraction of exosomes (Fig. 2b). As it follows from our data, CD117 is overexpressed in urinary exosomes of cancer patients with moderate-grade PCa (Gleason score 5 and 6) as compared to the patients without cancer and high-grade cancer patients. Due to well-established role of exosomes in cells-to-cell communication and cancer metastasis, such observation allows to speculate about the role of $CD117^+$ exosomes in prostate cancer progression. On the other hand, since the highest CD117 expression is registered for cancer patients with non-discriminative PSA value (patients 4 and 5), CD117 can be considered as exosomal marker—candidate for detection of early prostate cancer.

4 Conclusion

We demonstrated that hybridization of pre-enriched exosomes with anti-CD9 Dynabeads™ increases the sensitivity of the method. In our study, we observed no correlation between expression level of CD81, PSA level, and cancer grade (Gleason score). High level of CD117 expression in urinary exosomes was observed for patients with moderate-grade PCa. A further larger-scale study is required to confirm our observations.

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