Lectin-Induced Agglutination Method of Urinary Exosomes Isolation Followed by mi-RNA Analysis: Application for Prostate Cancer Diagnostic

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BACKGROUND. Prostate cancer is the most common cancer in men. Prostate-specific antigen has, however, insufficient diagnostic specificity. Novel complementary diagnostic approaches are greatly needed. MiRNAs are small regulatory RNAs which play an important role in tumorigenesis and are being investigated as a cancer biomarker. In addition to their intracellular regulatory functions, miRNAs are secreted into the extracellular space and can be found in various body fluids, including urine. The stability of extracellular miRNAs is defined by association with proteins, lipoprotein particles, and membrane vesicles. Among the known forms of miRNA packaging, tumour-derived exosome-enclosed miRNAs is thought to reflect the vital activity of cancer cells. The assessment of the exosomal fraction of urinary miRNA may present a new and highly specific method for prostate cancer diagnostics; however, this is challenged by the absence of reliable and inexpensive methods for isolation of exosomes.

METHODS. Prostate cancer (PC) cell lines and urine samples collected from 35 PC patients and 35 healthy donors were used in the study. Lectins, phytohemagglutinin, and concanavalin A were used to induce agglutination of exosomes. The efficiency of isolation process was evaluated by AFM and DLS assays. The protein content of isolated exosomes was analysed by western blotting. Exosomal RNA was assayed by automated electrophoresis and expression level of selected miRNAs was evaluated by RT-qPCR. The diagnostic potency of the urinary exosomal miRNA assessment was estimated by the ROC method.

RESULTS. The formation of multi-vesicular agglutinates in urine can be induced by incubation with lectin at a final concentration of 2 mg/ml. These agglutinates contain urinary...
INTRODUCTION

Prostate Cancer (PC)

Prostate cancer (PC) is the most commonly diagnosed male malignancy and the second leading cause of male cancer-related deaths [1]. Currently, testing for this disease heavily relies upon the detection of the androgen-regulated serine protease, prostate-specific antigen (PSA), in serum. However, elevated serum PSA levels may be associated with benign prostatic hyperplasia or gland inflammation, while "negative" PSA-reading (0–4 ng/ml) does not guarantee the absence of PC. Thus, the evaluation of new biomarkers able to track PC-development sensitively and specifically is required to improve PC diagnostics and management. Since the first discovery of cell-released membranous vesicles (exosomes) in biological fluids, including human urine [2,3], the diagnostic potency of urinary extracellular vesicles is now being explored for various pathologies, including both diseases of the urinary system [4–6] and systemic disorders [7]. A number of studies have revealed that specific alterations of the exosomal protein [8,9] and the miRNA [10] patterns are associated with PC. These results define the possibility of developing a new PC diagnostic tool that could supplement or even replace routine PSA assay.

Exosomes Purification

A major bottleneck in the development and application of exosomes-based diagnostic assays is the challenge of purification of exosomes; this requires time-consuming and instrument-based procedures. The currently existing protocols for exosome purification have been comprehensively reviewed [11]. In general, the principal approaches include ultracentrifugation [12], size-based isolation (ultra-filtration [13] or high-performance liquid chromatography [14]), precipitation by volume-excluding reagents, and affinity-based capture using exosome surface-specific ligands. Methods avoiding labour-intensive and time-consuming ultracentrifugation are being explored in kits produced by biotech companies (System Biosciences, Life Technologies, HansaBioMed, New England Peptide) that are available on the market. However, exosomes appear to present a relatively heterogeneous population in terms of their physical [15] and biochemical characteristics [16,17]. The specificity of isolation methods based on certain physical/biochemical property may result in isolation of certain exosomal fraction, not a total population. Moreover, biological fluids are complex solutions and may, obviously, contain other substances that overlap with exosome size-scales and molecular surface composition. Taking into account all these considerations, it is hard to see how any of the currently existing methodological approaches can guarantee both purity and maximal yield of exosomes isolation. Thus, new efforts are required to address this issue.

Lectin-Based Exosomes Agglutination

Most of the developed affinity-capture methods are based on the interaction of antibodies with proteins exposed on the exosomal surface, such as CD63, CD82, CD9, Alix, and EpCAM. In addition to the antibody-antigen interaction, the affinity of lectins to specific saccharide residues on the exosome surface is being explored. Lectins are plant- or animal-originating proteins binding various sugar moieties with a high specificity and a relatively low affinity [18]. As an example of practical application, binding of lectins to saccharide residues on the surface of circulating exosome was used to remove them from the plasma of cancer patients for therapeutic purposes [19]. Profile and biological function of the carbohydrates on exosomal surface is supposed to reflect cells of origin and to be implemented to interaction with recipient cells [20,21].

The surface of urinary exosomes is abundantly glycosylated. The first assessment of glycosylation profile of urinary micro-vesicles was done by flow cytometry and lectin microarray [22]. In that study, the glycosylation patterns of urine extracellular micro-vesicles showed little internal variation and were clearly distinct from those of glycosylated urinary proteins (Tamm-Horsfall protein, THP).
Another study aimed to identify lectins that are abundant on the surface of urinary exosomes and which could be used for their affinity-based isolation [23]. Thus, several lectins with specific affinity to N-Acetyl-Galactosamine (GalNAc), N-Acetyl-Lactosamine (LacNAc), N-Acetyl-Glucosamine, (GlcNAc), mannose, and galactose abundantly exposed on surface of urinary micro-vesicles were identified. The issue of THP contamination in context of lectin-based isolation of urinary exosomes was investigated as well [24]. However, no attempt to analyse the content of exosomes isolated from urine by means of lectin-binding has been reported so far.

miRNA Content of Urinary Exosomes

Extracellular miRNA isolated from urine may have PC-relevant significance. For instance, miRNA-483-5p was shown to be highly expressed in the urine of PC patients compared to the healthy controls; however, the authors of this report analysed the total urinary miRNA [25]. It is assumed that exosomal miRNAs may be a product of cancer cell activity and that it can have a distinct diagnostic significance compared to miRNA which is packaged in other forms [10]. The ratio and cellular source of various miRNA fractions, including Ago2 protein and lipoprotein coupled [26–28] in urine have still to be explored. We hypothesized that urine contains a very low amount of proteins and lipids that are capable of binding miRNA, and that exosomal miRNA presents the most abundant form. Based on this assumption, we were further expecting that lectin-induced exosomes agglutination can be applied for urinary exosomes isolation followed by miRNA assay. The present study aimed to prove this hypothesis and to set up a convenient and inexpensive method for urinary exosomes isolation and analysis. With this purpose in mind, we explored the process of the lectin-induced agglutination of exosomes produced by cancer cells in vitro, the RNA content and the miRNA pattern of these exosomes. Based on our findings, we developed a new method for the analysis of urinary exosomes isolated from patients with PC and from healthy donors. Using this method, we evaluated the diagnostic relevance of certain urinary exosome-derived miRNAs by a conventional receiver operating characteristic (ROC) assay.

MATERIALS AND METHODS

Cell Culture

Human prostate cancer (PC-3 and DU-145) and cervix carcinoma (HeLa) cell lines were purchased from American Type Culture Collection. Exosome-depleted FBS was purchased from SBI (USA), all other consumables for cell culturing including DMEM, PBS, penicillin, streptomycin, L-glutamine, and FBS were purchased from PanEco (Russia). For exosome isolation, cells were grown to 80–90% confluence as described [29] in serum-free DMEM for 2 days or in DMEM supplemented with 10% exosome-depleted FBS for 5 days.

Patient Samples

The study was conducted with approval of the Ethical Committee of N.N. Petrov Institute of Oncology. Written informed Consent was obtained from all subjects to use their urine and clinical data for research purposes. Data from 35 patients and 35 healthy controls are presented in Table I. Age-matched control group consisted of healthy individuals without clinical PC-relevant manifestations and with PSA level below 4 ng/ml. Morning urine was collected, without preceding digital rectal exam or prostate massage and before prostate biopsy or surgical removal of the prostate. Urine samples were centrifuged at 3,000g for 15 min (RT) and supernatant was collected and frozen at −80°C until use.

Exosome Isolation

From cell culture medium.
By differential centrifugation. For purpose of comparison, exosomes were isolated from cell culture media by classical methods as described [30]. Briefly, 100 ml of cell supernatants were centrifuged at 2,000g for 20 min to eliminate cells and then at 15,000g for 30 min to remove cell debris. Exosomes were pelleted by ultracentrifugation at 110,000g for 70 min at 4°C.

By lectin-induced precipitation. Medium was harvested and centrifuged as described above (2,000g for 20 min, then 15,000g for 30 min) to remove cells, cellular debris, and large membrane vesicles. Supernatant was incubated with Concanavalin A from Canavalia Ensiformis (Sigma–Aldrich, Russia) at a concentration of 1 mg/L (at +4°C, overnight) and then centrifuged again at 20,000g for 60 min. Pellet containing exosomes was dissolved in PBS and frozen at −80°C.

From urine.
By differential centrifugation. Upon thawing on ice, urine samples (13 ml) were supplemented with protease inhibitor cocktail, (Sigma–Aldrich) at recommended concentration. Cell debris and large vesicles were removed by centrifugation at 13,000g for 20 min. Supernatant was collected and ultra-centrifuged at
150,000g for 70 min. The pellet was dissolved in PBS and frozen at −80°C.

By lectin-induced precipitation. Upon thawing on ice, urine samples (40 ml) were supplemented with protease inhibitor cocktail and processed at +4°C. First centrifugation (20,000 g for 45 min) was performed to pellet cells, cellular debris and large vesicular structures. Supernatant was transferred into new tubes and incubated with Concanavalin A (Sigma–Aldrich) at a concentration of 2 mg/L (at +4 °C, overnight) and centrifuged again at 20,000 g for 90 min. Pellet containing exosomes was dissolved in PBS and frozen at −80°C.

Exosomes Characterization

Atomic force microscopy (AFM). Exosomes produced by cells in tissue culture were purified by ultracentrifugation and diluted in de-ionized water, adsorbed to freshly cleaved mica sheets, fixed with 0.5% glutaraldehyde, rinsed with de-ionized water and air-dried. The samples were scanned in the air by semi-contact method with a scanning microscope of Solver Bio series (NT–MDT, Russia) equipped with silicon test probe (type NSG01), with a characteristic stiffness of 5.5 N/m and a typical radius of curvature of the tip (less than 10 nm). The initial amplitude of scanning was set to 16 nA in current terms; SetPoint was adjusted to half of the amplitude. Scanning was performed with a frequency of 1.01 Hz. Images were processed using standard software package (Image Analysis Nova). Exosome agglutination was induced by incubation with lectins, PHA-M, Phytohemagglutinin or Concanavalin A (Sigma–Aldrich) at a concentration of 1 mg/L (at RT, 4 hr).

Dynamic light scattering (DLS). The Zetasizer Nano ZS (Malvern Instruments, UK) allows the analysis of particles with sizes ranging from 1 nm to 3 μm. Cells and cellular debris were removed by centrifugation from FCS-free cell media (15,000g for 30 min) and urine samples (20,000g for 45 min) before analysis. Nano-particle size distribution was analysed at 23°C according to the manufacturer’s instructions.

Western blot analysis. Cell pellets and exosomes isolated from urine or cell culture media were lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 1% sodium-deoxycholic acid, and protease inhibitors (Sigma–Aldrich) on ice for 1 hr and then centrifuged at 17,000g for 20 min. Protein extracts were normalized using the BCA Protein Assay Kit (Thermo Scientific, Russia), separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% skim milk in PBST, followed by incubation with primary antibodies (TSG101 (H-2) sc-22774, CD9 (H-110) sc-9148, elF2C/Ago1-4 (H-300) sc-32977) at dilution of 1:200 followed by incubation with goat anti-rabbit IgG-HRP (sc-2301) and developed using Luminol Reagent (sc-2048) (All from Santa Cruz). Blots were stripped by the western Blot recycling kit (Alpha Diagnostics, USA).

RNA Extraction and Analysis

RNA was extracted from cell pellets or from exosomes using RNA isolation Kit (BioSilica, Russia) using manufacture’s protocol based on conventional column-spin technology. RNA concentration and purity were confirmed by spectrophotometric ratio using absorbance measurements at wavelengths of 260/280 nm ratio with a NanoDrop 2,000 (Thermo Scientific, USA). Isolated RNA was further analyzed with the Agilent RNA 6000 Nano Kit using Tape Station 2,200 (Agilent Technologies Inc., USA).

Reverse-Transcription Quantitative Real-Time PCR

For miRNA quantification, cDNA was synthesized using miRCURY LNA Universal RT microRNA Polyadenylation and cDNA synthesis Kit (Exiqon, Denmark). Quantitative real-time polymerase chain reaction (qPCR) was performed using ExiLENT SYBR Green master mix (Exiqon, Denmark) on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Assayed miRNA and corresponding primers listed in Table II were designed by and purchased from Exiqon (Denmark). UniSp6 synthetic oligos was used as standard of absolute quantification while U6 snRNA and has-miR-191-5p were assayed and used for relative quantification.
Statistics

All statistical calculations were performed by MedCalc statistical software and by GraphPad software.

RESULTS

Isolation and Analysis of Exosomes From Cell Culture Medium

Cancer cell lines (HeLa, PC-3, DU-145) were cultured in medium supplemented with 10% fetal bovine serum depleted of exosomes for 5 days as described [29,30]. Exosomes were isolated by conventional ultracentrifugation and assayed by AFM (Fig. 1). The size of detected vesicles was in the range of 30–200 nm corresponding to well established diameter of exosomes. Serum starvation of cells for 5 days resulted in considerable enrichment of the media with vesicles (Fig. 1B). Incubation with lectins (PHA-M, Phytohemagglutinin or Con A, Concanavalin A) at final concentration 1.0 mg/L resulted in formation of large conglomerates detected by AFM (Fig. 1C). However, AFM visualizes surface-attached conglomerates of particles and does not reflect their size and spatial structure. Assuming nearly spherical form of observed conglomerates in suspension, their size was assayed by Dynamic Light Scattering. Thus, FBS free media was analyzed by DLS following two days of prostate cancer cell lines incubation. For PC-3 cells, particles with average diameter of 97.2 nm counted for 83.9% of scattered light intensity and were considered as cell-derived exosomes. Second peak referred to particles with average diameter 9.8 nm and reflected apparently protein complexes or small lipid micelles released by cells (Fig. 1D, red line). After incubation with Con A at conditions mentioned above shift of both peaks indicated an enlargement of particles size or formation of multi-particle conglomerates with average diameter equaled to 356.3 nm (Fig. 1D, green line). Similar results were observed for HeLa and DU-145 cell lines (data not shown). Assessment of fresh serum–free

![Fig. 1. Characterization of in vitro cancer cell—released vesicles. Exosomes from fresh media (A), conditioned serum free media after 5 days of prostate cancer cells incubation (B) and conditioned media incubated with Con A at concentration 1 mg/ml over 4 hr at RT (C) were observed under atomic force microscopy (AFM). (D) Size distribution of in vitro cell-released exosomes was estimated by Zetasizer Nano ZS (Malvern, UK) before (gray line) and after (black line) incubation with ConA at concentration 1 mg/ml.](image-url)
medium as a background did not reveal any light scattering objects. Thus, cancer cells cultured in FBS free medium produced two types of particles with size averaged around 10 and 100 nm, and lectins induced formation of large conglomerates of these particles. Combination of AFM and DLS analysis allowed us to assume that observed conglomerates may be formed by tens of particles and sedimentation of such conglomerates can be achieved by centrifugation with lower G-force than free exosomes.

In order to confirm that the observed particles are indeed exosomes, enrichment of exosomal protein markers in pelleted particles was assayed by western blotting. The second pellet obtained from medium after PC-3 cells incubation was enriched with exosomal markers (late endosomal membrane marker TSG101 and tetraspanins CD9) in comparison to total cellular lysate (Fig. 2, left). Keeping in mind the ultimate purpose of the study, we had to consider the presence of RISC components associated with miRNA in the cell medium. To exclude the possibility of contamination of exosomes-containing pellet with these complexes, protein lysates were assayed with antibodies against member of Argonaute protein family (Ago2). Presence of this protein was detected in whole cellular extracts, but was not detected in lectin-agglutinated and pelleted cell-derived particles (Fig. 3, left). Because lectin-induced conglomerates contain exosomes and do not contain detectable amount of RISC components, exosomes can be considered as a main source of RNA.

Next, RNA was isolated from lectin-agglutinated and pelleted exosomes. Sizing and quantization of exosomal RNA were done in comparison to cellular RNA (Fig. 3, left). Thus, ribosomal fractions (18S and 28S) were detectable in cellular RNA but were not observed in exosomal RNA fractions. Exosomal RNA was enriched with short molecules (200–2,000 nucleotides). To evaluate the pattern of miRNA in prostate cancer cells and in cell-derived exosomes, expression of 12 PC-associated molecules was assayed in PC-3 and DU-145 cell lines (Table II). Out of the 12 tested molecules, 6, and 7 miRNA specimens only were expressed at detectable levels in PC-3 and DU-145 cell lines, respectively. Patterns of detectable miRNAs in cells and cell-derived exosomes did not reveal any correlation (Fig. 4). For example, miR-301 was equally presented in cellular and exosomal fractions in both PC-3 and DU-145 cells. Cellular and exosomal levels of other miRNAs varied considerably. Moreover, ratios of cellular/exosomal fractions of miR-107 and miR-574 were opposite in the two tested cell lines revealing possible variability of exosomal miRNA sorting. MiR-375 was shown to be expressed in PC [31], however its functional activity seems to appear inside of cancer cells. This result is in line with recently published data [32]. Exosomes isolated from media by the conventional technique of differential ultra-centrifugation and by lectin-induced agglutination were assayed in parallel and their miRNA profiling gave almost overlapping results (Fig. 4, grey bars).

Our findings indicate feasibility of application of lectin-based exosome agglutination as an appropriate method for isolation of exosomes released by cancer cells in vitro. However, presence of glycosylated proteins that may bind miRNA (for instance Ago2, LPLD) may reduce applicability of this approach for the routine analysis of the most types of biological fluids. Since plasma proteins, including RISC compo-

Fig. 2. Western blot analysis of exosomal markers and Ago2 (eLF2C) proteins in PC-3 cell lines, exosomes from PC-3 cells, and urinary fractions. Analysis of PC-3 cell lysate and exosomes secreted from PC3 cells isolated via lectin-induced agglutination is presented on the left side. On the right side analysis of urinary pellet obtained by first centrifugation and supposed to contain cells, large vesicles and cellular debris (I pellet) and pellet obtained after incubation of supernatant with Con A followed by second centrifugation and supposed to be enriched by urinary exosomes (II pellet).

Fig. 3. Cellular and exosomal RNAs analyzed by Tape Station 2,200. (A) Total RNA isolated from PC-3 cells and (B) from exosomes secreted from PC-3 cells were isolated by lectin-induced agglutination. (C) Total RNA isolated from urinary pellet after first centrifugation at 20,000g assumed to contain cells and cellular debris, and (D) total RNA isolated from urinary pellet after incubation with Con A and second centrifugation at 20,000g is assumed to contain urinary exosomes.
nents, filtered into the primary urine are normally re-absorbed in the initial part of the proximal tubule, we supposed that urine is relatively depleted of miRNA-associated proteins. Urinary Tamm-Horsfall Protein (THP) is secreted in the thick ascending loop of Henle and is not associated with miRNA. Thus, lectin-induced co-agglutination of THP does not interfere with miRNA content of the exosomal conglomerates. We assumed that new approach can be applied for urinary exosome isolation and consequent profiling of exosomal miRNA.

**Isolation and Characterization of Urinary Exosomes**

In order to test our suggested approach, we applied new method for isolation of exosomes from urine of three healthy donors. Procedure included two centrifugation steps with lectin-induced exosomes agglutination between them (From Urine Section). It is assumed that cells exfoliated from urinary epithelium, cellular debris and large membrane vesicles are pelleted during the first round of centrifugation. Incubation of urinary supernatant with lectins results the agglutination of small vesicles (exosomes) into large agglutinates that can be pelleted by second centrifugation at moderate G-force.

Starting the procedure with 40 ml of urine, we obtained abundant pellet after the first centrifugation. Supernatant was incubated with lectin (Con A) at various concentrations (0.5, 1, 2, and 4 mg/L) overnight. Incubation with lectin at concentration ≥2 mg/L followed by centrifugation at 20,000g during 90 min yielded a barely visible pellet. Further increase of the centrifugation time did not increase the yield. Process of lectin-induced exosomes agglutination was assayed by DLS (Fig. 5). First, two types of urinary particles with size averaged about 10 and 100 nm were observed similarly to those in conditional media. Incubation with lectin resulted in formation of larger conglomerate with a size average of 600 nm. Thus, we detected two types of particles (about 10 and 100 nm) in both cultured media and urine, however lectins induced formation of larger aggregates in urine compared with conditional media. This observation may reflect more complex molecular composition of urine and implication of highly glycosylated THP into multi-particle complex. Second round of centrifugation at 20,000g for 90 min resulted in complete removal of large (600 nm) vesicles as was evaluated by DLS (not shown).

Presence of exosomal protein markers (TSG101 and CD9) and Ago2 protein was assayed by western blot in both pellets (Fig. 2, right). Certain amount of CD9 was detected in the first pellet that should mainly consist of cells, cellular debris and large vesicles. Both exosomal markers (TSG101 and CD9) were enriched...
in the second pellet that contains urinary exosome.
Ago2 was present in the first pellet only. It can be assumed that first centrifugation results in sedimentation of THP polymer that may entrap certain amount of exosomes. This may explain the detection of CD9 in the first pellet. At the same time, the presence of Ago2 protein may reflect sedimentation of cellular fragments or apoptotic bodies containing components of the RNA-induced silencing complex, RISC. Second centrifugation resulted precipitation of exosomes (Fig. 2, right). Comparative sizing of RNA in pellets after first and second rounds of centrifugation was performed as well (Fig. 3, right). Absence of 28S fraction, reduction of 18S fraction and large peak corresponding to RNA molecules length of 25–40 nt. may reflect presence of degraded cellular RNA in first pellet (Fig. 3C). The RNA pattern of second pellet was similar to pellets from in vitro cell-produced exosomes but had less distinct peaks in area from several tens to several hundred bases (Fig. 3 D). These results demonstrate that our new method of lectin-induced agglutination allows the isolation of urinary exosomes with specific pattern of RNA that is distinguished from the pattern obtained by cell lines in culture.

Taken together, these data indicate that lectin-induced agglutination can be applied for isolation of exosomes from urine. Next, applicability of this method for exosomal miRNA analysis with PC diagnostic purpose was addressed.

**Profiling of PC-Associated miRNA in Urinary Exosomes**

Urine from 35 prostate cancer patients and 35 healthy donors was collected and kept as described in Patient Samples Section. Patient characteristics are presented in Table I. Urine samples were thawed and processed through the first centrifugation (20,000g for 45 min), incubation with lectin (Con A) followed by second centrifugation (20,000g for 90 min) as described in From Urine Section. Twenty samples (10 from prostate cancer patients and 10 from healthy donors) were split and exosomes were isolated using both the lectin induced agglutination (40 ml) and differential centrifugation (13 ml) methods in parallel. Pellets containing exosomes were dissolved in 150 μl PBS and RNA isolation, reverse transcription and PCR were performed as described in sections RNA Extraction and Analysis and Reverse-Transcription Quantitative Real-Time PCR Sections and in accordance with Exicon’s protocols. PCR was performed in triplicates and means of Ct values were evaluated.

MiRNA that were included in the analysis were determined based on published data (referred in Table II) and included 12 miRNA that are over expressed in plasma or urine of PC patients. Most of the miRNA tested were shown to be over-expressed in plasma-derived circulating microvesicles [31] and their filtration through brood-urine barrier within the microvesicles could be assumed. From the list of 12 miRNA, five were detected in less than half PC patient’s urine samples tested. Those were excluded from further analysis (indicated by * in Table II). MiRNAs representation values were normalized to value of miRNA references (U6 snRNA and has-miR-191-5p) using standard approach (2 {CTreference-CTmiR of interest}). We found that miR-574-3p, miR-141-5p and miR-21-5p were presented at higher concentrations in the urine of patients with PC compared to control group (Fig. 6, left panels) with statistically significant difference (Table II).

In order to prove that both methods of exosome isolation does not influence miRNA profiling results, expression of 12 selected miRNAs was analyzed in 20 samples of exosomes isolated by differential centrifugation. In this experiment, tree miRNAs were detected in less than half PC patient’s urine samples tested and were excluded from analysis. From 9 miRNA analyzed, miR-574-3p, miR-141-5p, and miR-21-5p were found to be over-expressed in exosomes of 10 PC patients versus 10 healthy donors (Fig. 6, right panels). However, observed difference had lower statistical significance compared to analysis of lectin-induced conglomerates.

Finally, we wanted to determine the validity of three selected miRNAs as a diagnostic signature for PC and generated a specificity-sensitivity curve (Fig. 7). The presence of PC could be identified by assessment of urinary exosomal miRNA-574-3p (AUC ROC 0.85; 95%CI = 0.736-0.964), miR-141-5p (AUC ROC 0.86; 95%CI = 0.732-0.994), and miR-21-5p (AUC ROC 0.65; 95%CI = 0.477-0.814) with reducing sensitivity (0.71; 0.66; and 0.46 correspondently).

**DISCUSSION**

The implication of short regulatory RNA (miRNA) in carcinogenesis was first described in 2002 in context of B-cell chronic lymphocytic leukemia [33]. Since then, the number of studies on miRNAs and cancer has been increasing considerably, adding novel concept to the role of miRNAs in human tumors. Currently several miRNA-based cancer diagnostic tests are on the market (www.rosettagenomics.com and references therein) and many others are under development. In addition to the malignancy-associated alteration of cellular miRNA profile, the presence of tumor-derived miRNA in the serum of cancer patients was reported for haematological malignancies [34] and a number of solid tumors (reviewed

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in [35,36]). Meanwhile, the functional impact of circulating miRNAs in cancer development has been evaluated in experimental studies [37]. In addition, considerable progress has been made in gaining global insights into the functional importance and diagnostic potential of miRNA in PC [38].

It is now assumed that extracellular miRNA may exist in different forms, associated with the protein components of RISC, or loaded into extracellular vesicles (exosomes, microvesicles or apoptotic bodies) or into high-density lipoprotein particles. Given that cancer cells are reported to abundantly shed exosomes, it can be further hypothesized that cancer-associated circulating miRNA is packaged primarily in exosomes. Thus, the “fusion” of two concepts—miRNA and exosomes—has given rise to a new trend in cancer diagnostics [39]. However, the development and clinical application of exosomal miRNA-based tests are challenged by the lack of knowledge about exosome structure and biological functions, as well as absence of standards of preparation. Circulating exosomes appear to be a heterogeneous population in terms of cellular origin and physical [15] and biochemical characteristics [16,17]. Moreover, exosomes do not have absolutely and universally specific physical characteristics or biochemical markers that would allow us to purely distinguish them from other components of biological fluids. Thus, any of the existing methods for exosome isolation (discussed in Exosomes Purification Section) may be more or less selective for certain exosome fractions and contaminants. Despite the great progress made by biotech companies and the availability of exosome-purification kits on the market, the issue of exosome isolation remains challenging and compromises the results of exosomal miRNA analysis.

This problem was highlighted by a recent study on exosomal miRNA abundance and stoichiometry [40]. The authors in this study showed that the number of certain types of exosomal miRNA molecules is lower than the number of exosomes derived by standard preparations. The mean miRNA distribution was defined as 0.00825 molecules/exosomes. In other words, if miRNA was distributed homogenously across the exosome population, on average, over 100 exosomes would need to be examined to observe one copy of a given abundant miRNA. This stoichiometry of miRNA and exosomes suggests that most individual exosomes in standard preparations do not carry biologically significant amounts of miRNA. The authors interpreted their results by proposing several stoichiometric models of exosome/miRNA content, among which the existence of small fractions of exosomes with a specific miRNA content [40] may reflect the fraction of tumour-derived exosomes

Fig. 6. Difference of exosomal miRNA representation values between PC patients and healthy donors. Box-plot representation of indicated miRNA levels in urine samples from patients with prostate cancer and healthy group. Left panels show results of analysis of exosomes isolated by lectin-induced agglutination followed by centrifugation (35 PC patients vs. 35 healthy donors). Right panels show results of analysis of exosomes isolated by differential centrifugation (10 PC patients vs. 10 healthy donors). P value was calculated using the one-tailed Mann-Whitney U test in GraphPad software and statistical significance is indicated by *(P < 0.05) and **(P < 0.005).

Fig. 7. Diagnostic signature of exosomal urinary miRNAs. Receiver operating characteristic (ROC) curves for has-miR-574-3p, has-miR-141-5p, and has-miR-21-5p were created using MedCalc software (AUC ROC shown in brackets).
distributed within the total population of circulating exosomes. Assuming this model leads to two solutions: [1] the enrichment/depletion of certain exosome fractions during the isolation procedure will crucially change the results following miRNA analysis; [2] any approach to enriching the presence of cancer-derived exosomes during the isolation process will considerably improve the sensitivity following diagnostic miRNA assessment.

These issues may be addressed by the method described in this report. Various types of cells have a specific surface carbohydrate composition. For instance, specific patterns of surface carbohydrates were exhibited by various types of immortalized cancer cells [41]. Moreover, surface carbohydrate composition of cancer cell–released exosomes in vitro [20] as well as urinary exosomes/extracellular vesicles [22–24] were subjected to comprehensive studies recently. Further progress in this area may help to identify the specific surface carbohydrate profile of cancer-derived exosomes with respect to cancer-type or the source of the exosomes. In this context, the lectin-based isolation of exosomes may present an exceptionally attractive technology, because it would allow us to achieve specific compositions of exosomal agglutinates and desirable specificity of exosomes isolation by simple combining of various lectin species.

CONCLUSION

Within the present study, we confirmed the validity of lectin-based agglutination as a method for exosome isolation and consequent miRNA analysis. The results of the RT-qPCR assessment of miRNA from exosomes isolated by conventional or new methods were in good agreement. Moreover, these results are in line with the published data. For instance, hsa-miR-375 was shown to be expressed in PC-3 prostate cancer cells but not in exosomes released from PC3 cells [32]. This specific ratio of cellular to exosomal fractions of hsa-miR-375 was observed in our study in both PC-3 and DU-145 cells. The analysis of miRNA from exosomes isolated by lectin-induced agglutination from urine has also revealed the applicability of this approach for diagnostic purposes. Among detectable miRNAs, a significant over-representation of miR-574-3p, miR-141-5p, and miR-21-5p in the urinary exosomes of prostate cancer patients versus the control group was established. Thus, the miRNA pattern of exosomes isolated from urine by means of lectin-induced agglutination exhibits relevance to the early stages of the disease and may be adopted for diagnostic purposes.

Considering the relatively low cost and ease of implementation involved, the described method can be applied for clinical use and large research projects. Our further studies will aim to optimize this method by exploring different lectins and their combinations.

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