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An optimized procedure for exosome isolation and analysis using serum samples: Application to cancer biomarker discovery

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ABSTRACT

Exosomes are RNA and protein-containing nanovesicles secreted by all cell types and found in abundance in body fluids, including blood, urine and cerebrospinal fluid. These vesicles seem to be a perfect source of biomarkers, as their cargo largely reflects the content of parental cells, and exosomes originating from all organs can be obtained from circulation through minimally invasive or non-invasive means. Here we describe an optimized procedure for exosome isolation and analysis using clinical samples, starting from quick and robust extraction of exosomes with Total exosome isolation reagent, next isolation of RNA followed by qRT-PCR. Effectiveness of this workflow is exemplified by analysis of the miRNA content of exosomes derived from serum samples – obtained from the patients with metastatic prostate cancer, treated prostate cancer patients who have undergone prostatectomy, and control patients without prostate cancer. Three promising exosomal microRNA biomarkers were identified, discriminating these groups: hsa-miR375, hsa-miR21, hsa-miR574.

1. Introduction

Exosomes are RNA and protein-containing small vesicles (30–150 nm) constantly secreted by all cells in culture and in vivo, in both a normal and disease state [1–3]. Blood, urine, cerebrospinal fluid (CSF), breast milk, ascites fluid, amniotic fluid, bile, semen, saliva and sputum all contain 10^8–10^{13} of exosomes per milliliter of sample [4–6]. Depending on the cell or tissue of origin, many different roles and functions have been attributed to exosomes, including: intercellular communication, eradication of obsolete molecules, facilitation of the immune response, antigen presentation, programmed cell death, angiogenesis, inflammation, coagulation, dissemination of oncogenes from tumor cells and spread of pathogens such as prions and viruses from one cell to another [1–8]. Interest in exosomes range from their function in the body to more practical applications such as their use in biomarker development based on analysis of RNA and protein content. An ideal biomarker should be easily assayed with minimally invasive medical procedures but possess high sensitivity and specificity. Although many candidate biomarkers for various diseases have been proposed in the literature, very few have made their way to clinical use, and for certain types of cancer there are no reliable biomarker options.

For instance, it is well recognized that prostate cancer is over diagnosed and over treated [9–12]. Approximately 230,000 men were diagnosed with prostate cancer in 2014, however, almost four times as many had a biopsy performed after routine screening [13]. The identification of biomarkers that can assist with risk-stratification of patients would be highly beneficial, as their use will decrease the complications and morbidity related to unnecessary biopsy and prostatectomy. Exosomes, and in particular their miRNA, mRNA and lncRNA cargo, could potentially fill this void, providing biomarkers suitable for real-time detection and application to the management of prostate cancer patients.

The main advantages of miRNAs versus longer molecules (mRNA, lncRNA) include: (1) smaller number of sequences (<2000) and thus ease of analysis and (2) stability due to small size (~16–27 nt) and thus robustness of detection. Isolation of exosomal miRNA fraction from serum might be advantageous compared to analysis of whole serum in several ways; at the very least, it allows concentration of the sample, and the miRNA residing within vesicles is completely protected from endogenous RNases [5,14,15]. There is a pressing need within the research and medical communities for quick and easy methods of isolation of exosomes and their subsequent robust and sensitive analysis.

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Here we describe an optimized procedure for exosome isolation and analysis using clinical samples, starting from the extraction of exosomes with Total Exosome Isolation reagent to recovery of RNA with the Total Exosome RNA and Protein Isolation Kit, followed by analysis by qRT-PCR. Effectiveness of this workflow is exemplified by analysis of the miRNA content of exosomes derived from serum of the prostate cancer patients (with metastatic disease) vs patients that have undergone treatment (post prostatectomy) vs control patients without prostate cancer. Three promising exosomal microRNA biomarkers were identified, discriminating these groups: hsa-miR375, hsa-miR21, hsa-miR574. Following this fast and reliable workflow, disease-specific RNA signatures residing within the exosomes can be identified and used as biomarkers.

2. Materials and methods

2.1. Materials

Total exosome Isolation (from serum) reagent (Invitrogen), Total Exosome RNA and Protein Isolation kit (Invitrogen), 10× PBS (Ambion), nuclease-free water (Ambion), 100% ethanol, non-optical adhesive covers (Applied Biosystems), optical adhesive covers (Applied Biosystems), 384-Well PCR Standard Plates (Applied Biosystems), 96-Well PCR Standard Plates (Applied Biosystems), TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems), TaqMan Universal PCR Master Mix II, no UNG (Applied Biosystems), GeneAmp® PCR System 9700 (Applied Biosystems), 7900HT Fast Real-Time PCR System, SDS v2.3.

2.2. Blood samples

Blood samples were collected under an Institutional Review Board approved protocol. They were deidentified and separated into three groups. The first group included 8 patients with metastatic prostate cancer, with high levels of prostate-specific antigen (PSA > 500 ng/ml). The second group included 6 individuals who had undergone prostatectomy (at least 30 days prior), with PSA levels below the lowest level of detection (<0.1 ng/ml) using the Abbott Architect analyzer. Lastly, the third group included 10 patients without any history of prostate cancer and presenting to their physician for non-prostate conditions. Blood was collected in a gold-top Vacutainer blood collection tube (BD Inc., Franklin Lakes, NJ), then centrifuged at 1500*g*, for ten minutes to fractionate and the serum fraction was subject to exosome isolation.

2.3. Extraction of exosomes from serum using Total Exosome Isolation reagent

Serum samples (100 μl) were centrifuged at 2000*g* for 30 min to remove any cellular debris. The supernatant containing the cell-free serum was transferred to a fresh tube and each sample was combined with 1/5th volume (20 μl) of Total Exosome Isolation (from serum) reagent and then mixed well by pipetting up and down until a homogenous solution was formed. The samples were incubated at 4 °C for 30 min, then centrifuged at room temperature at 10,000*g* for 10 min. The supernatant was aspirated and discarded, and the exosome pellet was resuspended in 50 μl PBS buffer, then stored at 4 °C short term (1–7 days) or −20 °C for long term.

2.4. Sizing and quantification of exosomes with Nanosight® LM10 instrument

Exosomes purified from blood serum were diluted with PBS buffer (1–2 μl diluted 1000–10,000× in order to have the nanovesicle concentration in the working range for the instrument [2×10^8–8×10^8 particles/mL] and then quantified and sized using the Nanosight® LM10 instrument (Nanosight, UK), following the manufacturer's protocol. The LM10 uses a laser light source to illuminate nano-scale particles (10–1000 nm) which are seen as individual point-scatters moving under Brownian motion. The paths of the point scatters, or particles, are calculated over time to determine their velocity which can be used to calculate their size independent of density. The image analysis NTA software compiles this information and allows the user to automatically track the size distribution and number of the nanoparticles.

2.5. RNA recovery using the Total Exosome RNA and Protein Isolation Kit

The Total Exosome RNA and Protein Isolation Kit was utilized for recovery of RNA from the serum exosome samples. 48 μl of each sample was combined with 152 μl PBS, then 200 μl of 2× Denaturing Solution, vortexed to lyse, and then incubated on ice for 5 min. After incubation, 400 μl of Acid-Phenol:Chloroform was added to the mixture and vortexed for 30–60 s to mix. Samples were then centrifuged for 5 min at 10,000*g* at room temperature to separate the mixture into aqueous and organic phases. Once centrifugation was complete, the aqueous (upper) phase was carefully removed without disturbing the lower phase or the interphase and transferred to a fresh tube.

375 μl of 100% ethanol (1.25 volumes) was added to the 300 μl aqueous phase for each sample then vortexed to mix. The entire volume was placed onto a spin column in a collection tube then spun at 10,000*g* for 15 s to move the sample through the filter cartridge. Samples were then washed once with 700 μl Wash Solution 1 and twice with 500 μl Wash Solution 2/3 (centrifuged at 10,000*g* for 15 s for each wash). After washing, the filter was dried by spinning for an additional 1 min at 10,000*g*. The filter cartridge was transferred into a fresh collection tube and 50 μl of preheated (95 °C) elution buffer was applied to the center of the filter. Samples were centrifuged for 30 s at 10,000*g* to recover the RNA, then a second 50 μl volume of preheated (95 °C) nuclease-free water was applied to the center of the filter and centrifuged for 30 s at 10,000*g*. After the second spin, the eluate containing the RNA was collected and stored at −20 °C.

2.6. Reverse transcription and quantitative real-time PCR (qRT-PCR) analysis of the miRNA isolated from the exosomes

Reverse Transcription (RT) Master Mix was prepared for each sample using the TaqMan® MicroRNA Reverse Transcription Kit reagents and protocol (Applied Biosystems) with gene specific RT primers for 12 miRNA targets (miR16, miR20a, miR21, miR96, miR107, miR141, miR145, miR183, miR221, miR375, miR409, miR574). 12 μl of the RT Master Mix was added to corresponding wells in a 96-well plate, and 3 μl of each sample was added to the master mix. Plates were covered with adhesive non-optical cover and spun down to remove air bubbles, then placed into a 9700 thermocycler and incubated as follows: 16 °C for 30 min; 42 °C for 30 min; and 85 °C for 5 min. After RT, reactions were kept at 4 °C until use.

qPCR master mixes were prepared for each of 12 microRNAs by combining 5 μl of AB Universal PCR Master Mix II, 2.5 μl of nuclease-free water, and 0.5 μl of the 20× TaqMan Assay (hsa-miR-16 000391, hsa-miR-20a 000580, hsa-miR-21 000397, hsa-miR-96 000434, hsa-miR-107 000443, hsa-miR-141 000463, hsa-miR-145 002278, hsa-miR-183 002270, hsa-miR-221 000524, hsa-miR-375 000564, hsa-miR-409-3p 002332, hsa-miR-574-3p 002349). After mixing, 8 μl of each master mix was placed into wells in a 384-well plate (enough for duplicate reactions for each isolation replicate). 2 μl of each RT reaction was added in duplicate to the master