## **RESEARCH LETTER**

# mRNA fingerprint of early-stage clear cell renal cell carcinoma identified in urinary exosomes by mRNA sequencing

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**Introduction** Since their first identification by Pisitkun et al,<sup>1</sup> urinary exosomes (also referred to as small extracellular vesicles [sEVs]) became relevant to many research fields, especially those devoted to the discovery of molecular markers for urologic pathologies. The majority of sEVs derive from cells composing the kidneys and urinary tract, and carry heterogeneous molecular cargo indicative of the physiological and pathophysiological status of the urologic system. Urinary exosomes comprise probably the most valuable source of biomarkers associated with urologic diseases, as their molecular content is very well protected from enzymatic breakdown and has the lowest complexity compared with other urinary fractions and body tissues.<sup>2</sup> Recent research showed that exosomes released into the urine in the course of early-stage clear cell renal cell carcinoma (ccRCC) are characterized by a significantly different composition of lipids, RNAs, and proteins in comparison with sEVs from healthy individuals and patients with other urologic cancers.<sup>3-7</sup> These findings encouraged us to utilize urinary exosomes for uncovering novel coding transcripts associated with early-stage ccRCC.

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\* KM-B and AK contributed equally to this work. Clear cell renal cell carcinoma is a highly lethal urologic malignancy that accounts for about 4% of all cancer cases and over 2% of cancer deaths worldwide. If diagnosed at stage I or II, the disease can be successfully managed with surgery. Unfortunately, most cases of ccRCC are discovered incidentally during imaging tests, when one-third of patients had already developed locally advanced or metastatic disease.<sup>8</sup> The diagnosis of ccRCC is usually set postoperatively and there are no molecular screening tests available for its detection at the earliest, asymptomatic stages. In this study, we performed high-throughput sequencing of the mRNA content (mRNA-seq) of small urinary EVs from patients diagnosed with stage I ccRCC and chosen noncancer controls, that is, healthy individuals and patients with glomerulopathy, in order to find candidate mRNA species associated with nonadvanced stage of ccRCC.

Materials and methods Ethics statement The study was approved by the Institutional Ethics Committee of the Regional Specialist Hospital in Wroclaw, Poland (approval ID: KB/no. 9/2016). All participants provided written informed consent to be part of the study. The research was conducted in accordance with the Declaration of Helsinki.

**Description of participants** The study included healthy volunteers (3 men and 3 women; age range, 24–37 years), patients with primary stage I ccRCC (1 man and 3 women; age range, 66–79 years) as well as individuals with primary focal segmental glomerulosclerosis (FSGS; 3 men and 3 women; age range, 53–74 years) and primary immunoglobulin A nephropathy (IgAN; 4 men and 2 women; age range, 21–65 years).

Urine sample collection and handling The participants provided first-morning urine samples in sterile containers. The samples were obtained before surgery (in ccRCC patients) or renal biopsy (in IgAN and FSGS patients). They were centrifuged at 4300 × g for 30 minutes at room temperature and the aliquots of supernatant were stored at -80 °C. A summary of urine dipstick test results is included in Supplementary material, *Table S1*.





FIGURE 1 A – size distribution of the exosome population derived from urine supernatant from randomly selected individuals (overlay of 2 sequential measurements); B – electron microscopy image of small extracellular vesicles

**Isolation of urinary exosomes** Urinary exosomes were isolated from 5 ml of urine supernatant using a ExoQuick-TC PLUS Exosome Purification Kit (System Biosciences, Mountain View, California, United States), according to manufacturer's instruction.

**Exosome characterization** Particle size analysis Size measurements were performed using dynamic light scattering on a Malvern Zetasizer Nano-ZS ZEN3600 instrument (Malvern Instruments Ltd, Malvern, United Kingdom). Samples were irradiated with red light (wavelength,  $\lambda = 633$  nm) and intensity fluctuations of scattered light (detected at a backscattering angle of 173°) were analyzed to obtain an autocorrelation function. Data were acquired in manual mode (5 measurements in each 15 runs for 10 s).

Electron microscopy Small EV samples were placed onto 400-mesh formvar/carbon-coated copper grids. A 2% uranyl acetate solution was used as contrasting agent. After drying, the slides were imaged using a Hitachi H-800 Transmission Electron Microscope (TEM) (Hitachi, Tokyo, Japan) at a voltage of 120 kV. The analysis was conducted at the Laboratory of Microscopic Techniques, University of Wroclaw.

Exosome marker detection Exo-Check Exosome Antibody Arrays (System Biosciences) were utilized for assessing the expression level of 8 antigens typical of most exosomes and to check for the presence of cellular contamination (GM130). The antigen detection was performed according to manufacturer's recommendations. The protocol was conducted for 3 separate exosome preparations.

Silver staining The urine supernatant, and urine supernatant–derived exosomes were subjected to protein quantitation using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). A total of 20 µg of each specimen was loaded onto acrylamide gel. After separation, the proteins were silver-stained.<sup>9</sup> The staining procedure was performed for 3 different sample pairs.

### **mRNA sequencing workflow and statistical analysis** Total RNA was purified from the urinary exo-

somes using an Invitrogen's Total Exosome RNA and Protein isolation kit (Thermo Fisher Scientific). The RNA yield per sample was below  $1 \text{ ng/}\mu\text{l}$ . In order to extract the mRNA fraction, we used a Dynabeads mRNA DIRECT Micro kit (Thermo Fisher Scientific). Whole mRNA preparation was processed according to manufacturer's recommendations. The final mRNA-seq library was constructed from the mRNA transcripts using an Ion Total RNA-seq kit v2 (Thermo Fisher Scientific). cDNA samples were diluted to a final concentration of 0.05 ng/ml prior to emulsion polymerase chain reaction. After enrichment, a single library was loaded onto an Ion 316 Chip and sequenced (850 flows) on a Ion Torrent PGM sequencing platform (Thermo Fisher Scientific). The raw sequencing data were processed and analyzed on the Torrent Server using coverage analysis and the FileExporter plugin. In order to quantify and compare the mRNA profiles between the study groups we used the Qlucore Omics Explorer 3.5 software (Olucore AB, Lund, Sweden, license number: 9F6VT-890RV-S2VL8-LVME6). Trimmed mean of M-values was used as a normalization method. FunRich tool was used to assess the number of mRNAs shared between the study groups, and to calculate the percentage of transcripts that were found in the exosomes by previous studies (extracted from the ExoCarta and Vesiclepedia databases).<sup>10</sup> The "significant mRNAs" associated with early-stage ccRCC were analyzed in the context of their contribution to diseases using the DAVID functional annotation tool.<sup>11</sup>

**Results** Urine exosome characterization The size range of the isolated urinary vesicles was approximately 60 to 400 nm, which is typical of exosomes (FIGURE 1A). The sEV morphology was confirmed in TEM analysis (FIGURE 1B). Silver-dye staining of the protein content of the paired urine supernatant-derived sEV samples revealed significant differences in protein composition between the specimens (FIGURE 1C). Antibody arrays indicated that the samples were positive for the common sEV markers and free of cellular contamination (FIGURE 1D).



**mRNA-seq profiling** This study analyzed and compared the coding transcriptome of small urinary extracellular vesicles obtained from subjects with early-stage ccRCC, healthy individuals, and patients with noncancerous kidney diseases. The average number of usable sequencing reads obtained for particular groups equaled 467 084 for ccRCC libraries, 463 489 for healthy controls, 729440 for FSGS, and 572289 for IgAN samples. The RNA-seq identified 731, 1509, 939, and 871 mRNA transcripts in ccRCC, healthy control, FSGS, and IgAN samples, respectively (the cutoff threshold for the number of reads per gene was set to >10) (Supplementary material, Table S2). Among the mRNAs identified across all groups (and all samples), 420 sequences were found to be common for each group (Supplementary material, Table S2). The mRNA pools defined for the ccRCC, healthy control, FSGS, and IgAN groups, respectively, included 15%, 24%, 23%, and 21% of transcripts that were previously detected in exosomes obtained from various biological fluids and tissues (FunRich analysis).<sup>10</sup> Quantitative comparative analysis of the overlapping transcriptomic fractions between the ccRCC and reference groups allowed to extract an mRNA cluster "highly" specific for early-stage ccRCC. Five coding transcripts composing the set, namely, NME2, AAMP, CAPNS1, VAMP8, and MYL12B were found



FIGURE 1 C – a heatmap presenting the relative expression level of the exosome and nonexosome markers (GM130) (unshaded area, lack of expression; light gray, low expression; dark gray, high expression); D – representative silver staining of the proteins isolated from paired urine supernatant–derived exosome samples; E – principal component analysis plot illustrating the "degree" of similarity between experimental specimens based on the 5-gene signature expression profile; F – a heatmap depicting the 5-gene expression pattern across all experimental samples Abbreviations: ccRCC, patients with clear cell renal cell carcinoma; FSGS, patients with focal segmental glomerulosclerosis; IgAN, patients with immunoglobulin A nephropathy

to be significantly and strikingly underrepresented in urinary exosomes of ccRCC patients (average fold change, 25–56, Q < 0.0007) in relation to other samples (FIGURE 1E and 1F).

Discussion To date, only several studies attempted to identify biomarkers specific for early-stage ccRCC by examining the molecular constituents of urinary exosomes.<sup>3-7</sup> The study by De Palma et al<sup>6</sup> was the first and only one to examine total transcriptome of urinary sEVs derived from ccRCC patients and identified a "3-mRNA cluster" indicative of early stage ccRCC. Unfortunately, the 3 transcripts showed only a 2-fold or less difference in the expression between ccRCC and healthy controls.<sup>6</sup> This research utilized microarrays, which are known to be characterized by lower dynamic range and sensitivity compared with RNA-seq. Our analysis is the first to use the mRNA-seq protocol to uncover urinary exosome mRNAs associated with early-stage ccRCC. The exact role of the coding transcripts, that is, NME2, AAMP, VAMP8, and MYL12B (except *CAPNS1*) in ccRCC biology remains unknown.<sup>11,12</sup>

**Conclusions** We presented a set of 5 novel mRNAs specific for stage I ccRCC which were identified for the first time by a powerful mRNA sequencing strategy. The main limitation of our

study is the small sample size, thus the proposed cluster requires further validation in larger cohorts of individuals representing a wide spectrum of health status, that is, healthy individuals as well as patients at various stages of ccRCC and non-ccRCC urological cancers, and those with noncancerous urologic diseases.

#### SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

#### **ARTICLE INFORMATION**

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#### CONFLICT OF INTEREST None declared.

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