A Comprehensive Proteomics Analysis of Urinary Extracellular Vesicles Identifies a Specific Kinase Protein Profile as a Novel Hallmark of Medullary Sponge Kidney Disease

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INTRODUCTION

Recent advances in the high-throughput technologies and data analysis have led to better understanding of the biological processes involved in several kidney disorders, including the medullary sponge kidney (MSK) disease, a rare congenital malformation characterized by dilation of the collecting ducts in the renal papillae, urinary acidification and concentration defects, cystic anomalies of precalyceal ducts associated with a high risk of nephrocalcinosis, and recurrent kidney stones.

Our research group, analyzing both the whole urinary proteome profile1,4 and the protein content of urinary extracellular vesicles (microvesicles and exosomes),5,6 has previously identified specific biological fingerprints of MSK able to discriminate this disorder from other clinical conditions including idiopathic calcium nephrolithiasis (ICN)1 and selected potential biomarkers that could allow earlier diagnosis of MSK avoiding expensive and invasive diagnostic approaches.

Laminin subunit alpha 2 was one of the promising identified proteins. This is a major component of the extracellular matrix that may have a role in cystic dilatation of the precalyceal ducts and in renal tubular epithelial cell polarization.53,54

Instead, the proteomic analysis of urinary extracellular vesicles5,6 was able to differentiate patients with MSK from those affected by autosomal dominant polycystic kidney disease.6 Analysis of the protein content of urinary extracellular vesicles of patients with the 2 disorders suggested a different mechanism of cystogenesis and probably confirmed earlier reports indicating MSK could be an inborn malformation.57

However, even with the reports of these literature, the physiopathologic mechanism associated with this disease is not fully understood. Therefore, we reinterrogated proteomic raw data previously obtained from urinary microvesicles of MSK and ICN5 applying updated bioinformatic methodologies to identify a specific urinary extracellular vesicle proteomic fingerprint differentiating patients with MSK from those affected by ICN (controls).

RESULTS

The protein composition of urinary exosomes and microvesicles of patients with MSK and ICN was determined by mass spectrometry (details in the Supplementary Methods). We identified 2978 proteins in the exosomes and microvesicles isolated from urine of patients with MSK and ICN (Supplementary
Among these, 2491, 2456, 2471, and 2190 were classified in the exosomes or microvesicles of MSK or ICN samples, respectively. In addition, 1774 proteins (60.5%) overlapped in all samples, whereas only 119 (4.1%) and 25 (0.9%) were exclusive for exosomes and microvesicles of MSK samples and 57 (1.9%) and 83 (2.8%) were exclusive for exosomes and microvesicles of ICN samples, respectively (Supplementary Figure S1).

Total identified proteins were classified according to the cellular component, molecular function and biological process of the gene ontology signatures. Using these classification and other gene ontology annotations extracted from UniProt database (www.uniprot.org), a gene ontology enrichment analysis was performed. This analysis showed an enrichment of 57 signatures (Supplementary Table S3). Interestingly, the most discriminative signature in the extracellular vesicles able to differentiate the 2 study groups was that including kinases (n = 82 identified proteins) (Figure 1a and Supplementary Table S3). Among the 82 identified kinases, 62, 47, 68, and 71 were identified in the exosomes or microvesicles of the MSK or ICN samples, respectively.

Noteworthy, 37 of 82 kinases (45.1%) overlapped in all samples, whereas only 2 of 82 (2.4%), 0 of 82 (0%), 5 of 82 (6.1%), and 4 of 82 (4.9%) were exclusive for the exosomes or microvesicles of the MSK or ICN samples, respectively (Supplementary Figure S2). Moreover, 34 of 82, 17 of 82, and 14 of 82 were highlighted in analysis of variance test and t test in the comparison of exosomes or microvesicles of MSK and ICN, respectively (Supplementary Figure S2B and Supplementary Table S4).

Identified kinases in the exosomes or microvesicles of the MSK or ICN samples were visualized by means of the interactive web application CoralTree (Figure 1b).
and Supplementary Figures S3 and S4) and volcano plot (Figure 2a and Supplementary Tables S3 and S4).

The expression profile of these proteins in the exosomes and microvesicles of the MSK and ICN samples, after Z-score normalization, was visualized in a heatmap diagram (Figure 2b).

Finally, according to several statistical algorithms, ephrin receptors (EphA1, EphB3, and EphB6) resulted the most promising kinase biomarkers for differentiating the 2 study groups (Figure 2b). Enzyme-linked immunosorbent assay (ELISA) validated these results (Supplementary Figure S5).

**DISCUSSION**

In this study bioinformatic analysis revealed that several kinases were able to differentiate MSK from ICN (control) and that 3 ephrin receptors (EphA1, EphB3, and EphB6) resulted the most significantly downregulated proteins in MSK versus ICN.

Ephrin receptors, belonging to the largest subfamily of receptor tyrosine kinases, are subdivided based on sequence similarity into an A subclass (EphA1–EphA8) and a B subclass (EphB1–EphB4, EphB6). Their ligands, the ephrins, are cell surface-bound proteins classified into 2 subfamilies based on their mode of membrane attachment: ephrins A (A1–A6) and ephrins B (B1–B3). S10

Ephrin receptors and ephrins are expressed in almost all tissues of a developing embryo and are involved in a wide array of developmental processes (including cardiovascular and skeletal development, axon guidance, modulation of cell adhesion and migration). S11

These biological processes, whether deregulated, may be involved in the pathophysiology of the MSK disease. In fact, based on available literature, MSK could belong to congenital anomalies of the kidney and urinary tract. S2, S12, S13 Its association with several developmental defects in other organs S2 suggests that this defective embryogenic step could be shared by the various organs involved. S14–S16

In addition, as recently reported, S7 tyrosine kinases and ephrin ligands may regulate kidney cytoarchitecture once development is completed. Immunolocalization
mutations in GDNF gene, S21 we cannot exclude that this disease.

tation, cystic dilations of precalyceal ducts, and extra-

kinases (e.g., MAPK) could orchestrate cell prolifera-

ephrins (in particular, EphB3 and EphB6) and other

including laminins (mainly laminin 2) together with

deregulated in MSK. Several biological elements

demonstrated that a complex biological machinery is

GDNF/RET deregulation may in

therapeutic targets.

may represent future disease biomarker and potential

selected proteins (mainly EphA1, EphB3, and EphB6)

associated with this rare kidney disorder. Finally, the

expression.

Kidney International Reports (2022) 7, 1420–1423

Because 8% of patients with MSK in Italy have

mutations in GDNF gene, S21 we cannot exclude that the

GDNF/RET deregulation may influence kinase

expression.

Although additional studies are necessary to define

the impact of kinases and ephrin signaling in MSK, we

can postulate that kinase down-regulation could be a

characteristic of this pathology and represent a new

and previously unrecognized biological mechanism

associated with this rare kidney disorder. Finally, the

selected proteins (mainly EphA1, EphB3, and EphB6)

may represent future disease biomarker and potential

therapeutic targets.

DISCLOSURE

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary References.

Figure S1. Venn diagram of total proteins identified and

gene ontology annotation enrichment analysis.

Figure S2. Venn diagram of total kinases identified and

Venn diagram of statistically significant proteins.

Figure S3. Kinome tree of exosomes isolated from urine of

MSK and ICN samples.

Figure S4. Kinome tree of microvesicles isolated from

urine of MSK and ICN samples.

Figure S5. ELISA validated proteomic data.

Table S1. List of total protein identified in exosomes and

microvesicles isolated from urine of MSK and ICN patients.

Table S2. List of total proteins identified in exosomes and

microvesicles isolated from urine of MSK and ICN patients

with their statistics.

Table S3. Gene Ontology enrichment in exosomes and

microvesicles isolated from urine of MSK and ICN patients.

Table S4. List of total kinases identified in exosomes and

microvesicles isolated from urine of MSK and ICN patients.

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