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RAPID COMMUNICATION

Proteomic analysis of urinary exosomes reveals ferroptosis-associated proteins are involved in diabetic nephropathy

Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes mellitus and a major cause of end-stage renal disease (ESRD). The pathogenesis of DN is unknown, but it is closely related to disorders of glucolipid metabolism, abnormal hemodynamics, chronic inflammatory response, oxidative stress, and genetics. Once DN develops into ESRD, it is often more difficult to treat than ESRD caused by other kidney diseases. Therefore, a deeper knowledge of the pathophysiological mechanisms of DN and the discovery of candidate markers for early diagnosis are mandatory. Exosomes secreted by renal cells can regulate a series of pathophysiological processes such as translation and transcription by releasing miRNAs or proteins, thereby regulating the biological functions and phenotype of recipient kidney cells. It has been found that miRNA secreted by urinary exosomes in patients with DN is involved in the occurrence and development of DN and may become biological markers.[1] However, studies on exosome proteins are relatively few and lack validation in humans. In the present study, we identified an altered pathway in urinary exosomes from DN patients for the first time. Notably, the altered ferroptosis-related proteins may represent novel candidate markers for early progression of disease and/or early treatment effects.

Urine exosomes isolated from six type 2 diabetes (T2D) patients without DN (called group A) and stage III DN patients (called group B) were analyzed by mass spectrometry (label-free). The clinical characteristics of the subjects were shown in Table S1. Urine albumin creatine ratio (UACR) and transferrin (TF) levels of group B were significantly increased compared with those of group A. No differences could be found in gender, age, course of DM, body mass index (BMI), haemoglobin A1c (HbA1c), creatinine (Cre), urea nitrogen (BUN), uric acid (UA) or estimated glomerular filtration rate (eGFR) levels between group A and group B. The ultrastructure of exosome preparations was examined by electron microscopy and westing blot. As shown in Figure S1A, exosome contained lipid bilayer bound membranes, with size distributions peaking at about 40—100 nm. In addition, immunoblotting was performed to detect the expressions of exosome-marker proteins (CD9, CD63, and TSG101), which were significantly expressed in exosome preparations (Fig. S1B).

To determine exosome protein composition, extensive mass spectrometry proteomics analyses were performed, resulting in the identification of a total of 3234 proteins in both groups. The distribution of significantly differential proteins among the three groups was observed using fold change (FC, Group B/Group A) > 2 or FC < 0.5 and $P < 0.05$ as the criterium to screen upregulated or downregulated proteins. The significantly differential proteins were plotted using a volcano plot (Fig. S2A) and a hierarchical clustered heatmap (Fig. S2B). A total of 83 proteins were differentially expressed, including 56 upregulated and 27 downregulated proteins. Subcellular localization analysis showed that the differential proteins were predominantly localized in extracellular, plasma membrane, cytosol and nucleus (Fig. S2C).

In order to understand the functional significance of differential expressed proteins, they were classified according to biological process, molecular function, and cellular components. Differential proteins were classified according to their ontology, which was determined by their gene ontology (GO) annotation terms (Fig. 1A). The annotated biological processes of the proteins were mainly involved in many different biological functions, such as regulation of signaling receptor activity, cellular response to iron ion, regulation of iron ion transport and so on.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was used to analyze and calculate the protein enrichment degree of different

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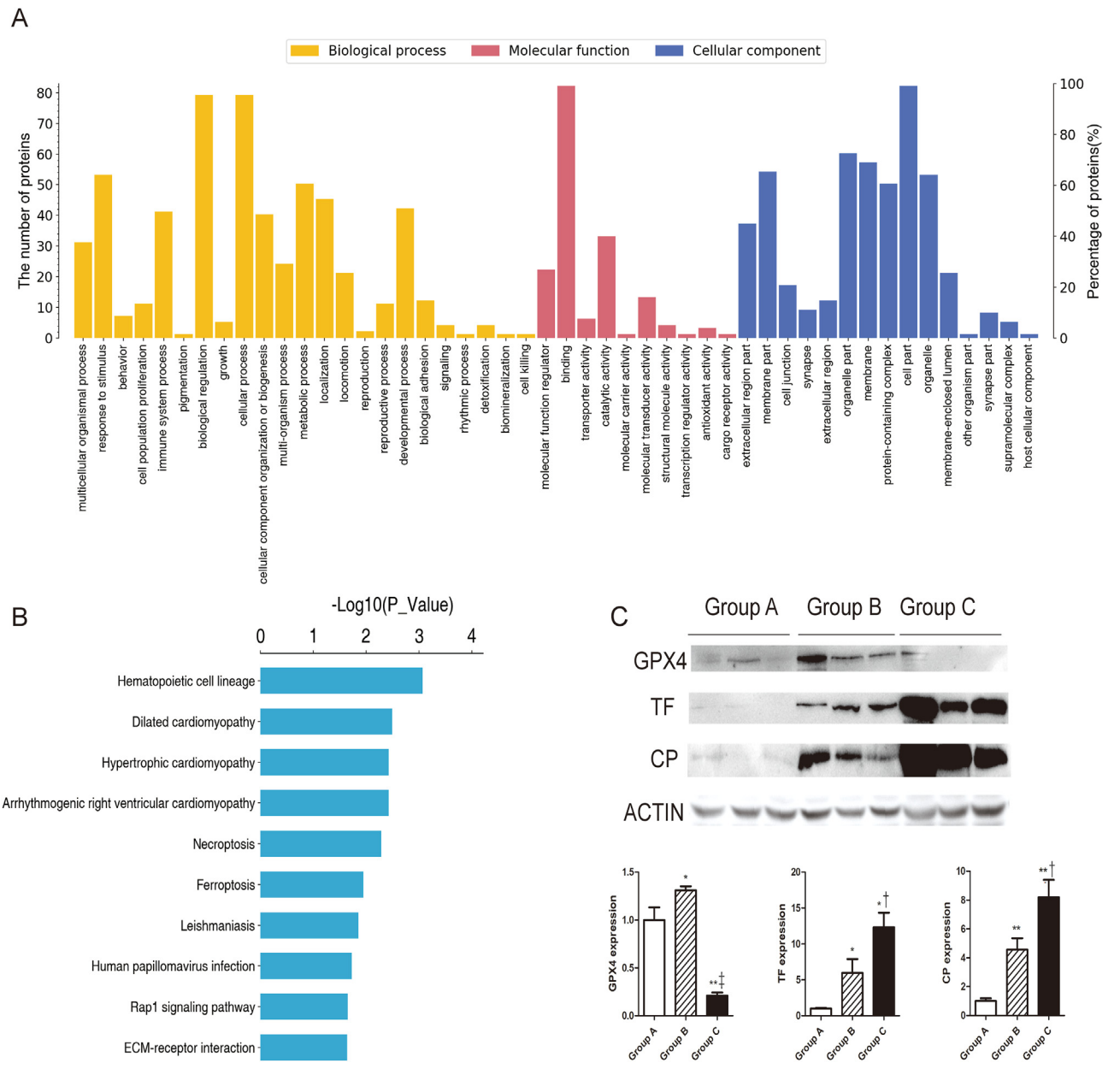

Figure 1 Bioinformatics analysis for mass spectrometry and validation by Western blotting. **(A)** Top 10 enriched GO cellular components, molecular function, and biological process. **(B)** Top 10 enriched KEGG pathways. **(C)** Selectively differentially expressed proteins were validated by Western blotting. Group A: T2D without DN. Group B: stages III DN. Group C: stages IV DN. *$P < 0.05$ vs. Group A, **$P < 0.01$ vs. Group A, $^{\dagger}P < 0.05$ vs. Group B, $^{\ddagger}P < 0.01$ vs. Group B. $n = 6$ in each group.

pathways. The top 10 significantly differentially expressed proteins were mainly involved in metabolic and signal transduction pathways such as necroptosis, ferroptosis, Ras-proximate-1 signaling pathway and extracellular matrix—receptor interaction (Fig. 1B).

Ferroptosis is a regulatory process of cell death, including iron metabolism, thiol regulation processes and lipid peroxidation. Recently, research found that Acyl-CoA Synthetase Long Chain Family Member 4, a marker of ferroptosis, is mainly expressed in renal tubules, and iron content in DN models increased significantly.[2] Treatment with ferrostatin-1, as a ferroptosis inhibitor, can alleviate renal pathological changes in DN mice models.[3] In this study, three ferroptosis-associated differential proteins, namely, Glutathione Peroxidase 4 (GPX4) (FC = 2.1, $P = 0.049$), TF (FC = 9.5, $P = 0.003$) and Ceruloplasmin (CP) (FC = 4.3, $P = 0.039$), were found to be upregulated in DN patients. And then, they were detected by Western blot in exosomes from all subjects in the two groups to validate proteomic data. In agreement, compared to group A, the specific staining for these proteins showed moderate to high staining intensity in group B (Fig. 1C).

GPX4 is one of the most important antioxidant enzymes, and its expression is significantly decreased in the cells and kidneys of DN models.[3] By contrast, in this study, we found that GPX4 was highly expressed in patients with stage III DN

(group B) compared with DM patients without DN (group A). To get more insight into GPX4 identified in DN, another stage IV DN patients (called group C) were recruited for urine exosome isolation and immunoblotting. As shown in Figure 1C, compared to group B, GPX4 was downexpressed in patients with stage IV DN (group C), while TF and CP were dramatically upregulated. TF is a glomerular marker that increases significantly with the progression of glomerular diffuse lesions. In the initial stage of kidney diseases, TF may be excreted before microalbuminuria occurs and it can be easily excreted in the advanced stages of kidney damage. CP is a copper-carrying metal enzyme, acts as an antioxidant through its ferroxidase activity. However, in the case of elevated oxidative stress, it may induce the formation of reactive oxygen species and the oxidation of low-density lipoprotein by providing free copper ions as promoters. Previous studies have shown that serum CP level is positively correlated with proteinuria in patients with T2D, and serum CP is an independent predictor of progression of DN.[4] Recently, Tsvetkov P et al found that copper-dependent regulated cell death differs from known death mechanisms in human cells and relies on mitochondrial respiration.[5] CP is an important copper-carrying metalloenzyme, but whether it is involved in copper induced cell death and thus copper induced cell death in the development of DN requires further study.

In conclusion, our study, for the first time, provided evidence that ferroptosis is involved in the occurrence and development of human DN. The expression of GPX4 was compensatory increased in urine exosomes of patients with early stages of DN, and TF and CP were increased with the aggravation of DN. TF and CP may be the initial factor of ferroptosis in the development of DN. These proteins may be the novel candidate markers for early progression of DN. This study may provide a new insight into the study of the mechanism of DN from the perspective of ferroptosis.

Conflict of interests

The authors declare that they have no competing interests.

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Ethics declaration

All investigation were conducted according to the principles expressed in the Declaration of Helsinki and approved by the ethics committee of Zhoupu Hospital (Approval number: 2022-C-012).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.101138.

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