Differential expression study of circular RNAs in exosomes from serum and urine in patients with idiopathic membranous nephropathy

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Abstract

Introduction: The aim of the study was to further explore the pathogenesis of idiopathic membranous nephropathy (IMN), gene-sequencing was used to analyze the differentially expressed circRNAs in exosomes of patients with IMN, which may lay the foundation for the research of circRNAs as a new class of exosome-based IMN diagnosis biomarkers.
Material and methods: Ten patients with IMN and ten normal controls were recruited as experimental subjects in our study. The exosomes were extracted from the collected serum and urine. Then, pure circRNAs were extracted from the exosomes with a series of enzymatic reactions. Afterwards, the significantly differentially expressed circRNAs were chosen by the method of gene-sequencing.
Results: Compared with normal controls, the circRNAs were reduced in the exosomes from serum of patients with IMN, which mostly originated from intron gene regions. Meanwhile, a total of 89 circRNAs were significantly differentially expressed, which were also mostly derived from intron gene regions, including 49 up-regulated and 40 down-regulated genes. However, the species were increased in the exosomes from the urine of patients with IMN compared to normal controls, and they mainly originated from exon gene regions. Simultaneously, 60 circRNAs were significantly differentially expressed, which primarily belonged to intron gene regions, including 54 up-regulated and 6 down-regulated regions.
Conclusions: The significant differential and specific expression of circRNAs in the exosomes from patients with IMN were observed. For example, MUC3A, which originated from chr7:100550808|100551062, could be considered a potential diagnostic biomarker of IMN. Furthermore, these figures may be used as a reference or supplement in the research of the pathogenesis of IMN.

Key words: exosome, circular RNA, idiopathic membranous nephropathy, gene sequencing.

Introduction

Idiopathic membranous nephropathy (IMN) is the most common cause of adult nephrotic syndrome. Approximately 25% to 40% of adult primary nephrotic syndrome cases have IMN. Idiopathic membranous nephropathy is also the most common pathologic type of glomerular disease, and IMN has a longer disease course. The prognosis of IMN var-

ies [1]. The pathologic features of IMN are a high number of immune complexes deposited in the glomerular basement membrane on the epithelium side.

The exosome has a double layer plasma membrane structure. Its diameter is approximately 30–100 nm, and it carries a rich protein, mRNA and microRNA. Exosomes are released to the extracellular microenvironment by the cells [2, 3]. They can be released from fibroblasts, dendritic cells, tumor cells and other cells; they are widespread in the urine [4], peripheral blood, saliva, cerebrospinal fluid, amniotic fluid, ascites and other body fluids [3, 5]. Therefore, we can detect exosomes and their contents from tissue, cells and body fluids to diagnose and clinically treat the disease, especially kidney disease. Miranda *et al.* [6] observed exosomes of renal tubular epithelial cells, podocytes, collecting duct cells and leap cells by transmission electron microscopy, which showed that almost all kidney inherent cells could secrete exosomes. In addition, the authors found that the components of exosomes were different in normal physiological conditions and disease conditions even for the same tissue or body fluid [7]. Previous studies have shown that the contents of exosomes have a characteristic change in acute kidney injury [8], IgA nephropathy [9], diabetic nephropathy [10], renal tubular acidosis [6], polycystic kidney [11] and other kidney diseases. The findings suggested that exosomes can be used as specific markers for early disease diagnosis.

Recent studies have shown that circRNAs can be used as biomarkers for the diagnosis and efficacy of a variety of clinical diseases, such as atherosclerosis [12], neurological diseases [13–15], diabetes [16], tumors [17–19] and more. In addition, because of the high stability of the circRNAs and the difficulty of degrading them by exonuclease, we can easily obtain circRNA from body fluid [12]. Based on the above characteristics, circRNAs show great potential to regulate human disease genes [20], making them a current research focus. In 2005, Huang found many exosomes in human serum and discovered that there is a difference in the exo-circRNA between colorectal cancer and normal human serum [21]. The authors speculated that circRNAs could be used as a new biomarker for cancer diagnosis. This discovery renewed people's awareness of circRNAs and exosomes because the authors had linked two emerging areas and further demonstrated the importance of circRNA and exosomes in organisms [21].

In this study, we evaluated circRNAs of exosomes. We compared the expression of circRNAs in the exosomes of serum and urine in patients with idiopathic membranous nephropathy and normal healthy controls by gene sequencing. Then, we screened out the differential expression of circRNAs and performed further analysis. The rich data from the analysis provide insight into the pathogenesis of IMN and a solution for future diagnosis and treatment.

Material and methods

Patient assessments and classifications

The study protocols and consent forms were approved by the Second Clinical Medical College (Shenzhen People's Hospital) of Jinan University and adhere to the Helsinki Declaration guidelines on ethical principles for medical research involving human subjects. Written informed consent was obtained from all participants. Ten IMN patients who had never been treated with glucocorticoids or other immunosuppressive drugs were recruited for this study. In addition, we chose 10 healthy subjects as controls (Table I).

Inclusion and exclusion criteria

The inclusion criteria were as follows: IMN patients were hospitalized at Shenzhen People's Hospital nephrology department from November 2015 to October 2016. Renal biopsy confirmed that their pathological type was idiopathic membranous nephropathy and their kidney function was normal before and after admission.

The exclusion criteria were as follows: 1) patients with abnormal renal function based on increased urea nitrogen or creatinine; 2) secondary nephrotic syndrome patients, such as those with hypertensive nephropathy, diabetic nephropathy, lupus nephritis, and hepatitis-related nephritis; and 3) renal pathology results confirming membranous nephropathy, but the patient has co-occurrence of another disease that can cause renal damage, such as hypertension, diabetes, systemic lupus erythematosus, hepatitis B and others.

Collection of serum and urine specimens:

1) All patients met the inclusion criteria and they were prohibited from eating or drinking the night before specimens were collected.

Table I. Clinical characteristics of IMN patients and normal controls

Group	IMN group	NC group
Age [years]	38.61 ±11.21	35.14 ±12.13
Sex (M/F)	7/3	7/3
Serum creatinine [μmol/l]	74.9 ±23.6	63.8 ±20.4
Proteinuria [g/24 h]	2.50 ±1.28	0.08 ±0.03
Serum albumin [g/l]	34.04 ±8.79	42.57 ±3.16
PLA2R (%)	60% (6/10)	0% (0/10)

NC group – normal control group.

2) Venous blood was collected the next morning from elbow vein blood and then kept at 37°C to promote coagulation.
3) Samples were centrifuged for approximately 10 min at 3000 rpm.
4) Approximately 2–3 ml of the upper layer of liquid was absorbed into the EP tube, which was marked with identification information (date, number, etc.) and then stored at –80°C.
5) At the same time, the patient's first morning urine (approximately 100 ml) was collected into a centrifuge tube, which was marked with identifying information (date, number, etc.) and then stored at –80°C.

Exosome isolation

Exosomes were isolated by the polymer formulation method [22] from blood serum using an ExoQuick reagent precipitation kit (System Biosciences, SBI, Mountain View, CA) according to the manufacturer's protocol. This exosome isolation method has been well validated with other techniques, including electron microscopy [22, 23]. All exosomes were stored at –80°C immediately after isolation until further analysis. The total protein concentration of the isolated exosomes was determined using the standard Bradford protein assay (Bio-Rad, Richmond, VA, USA).

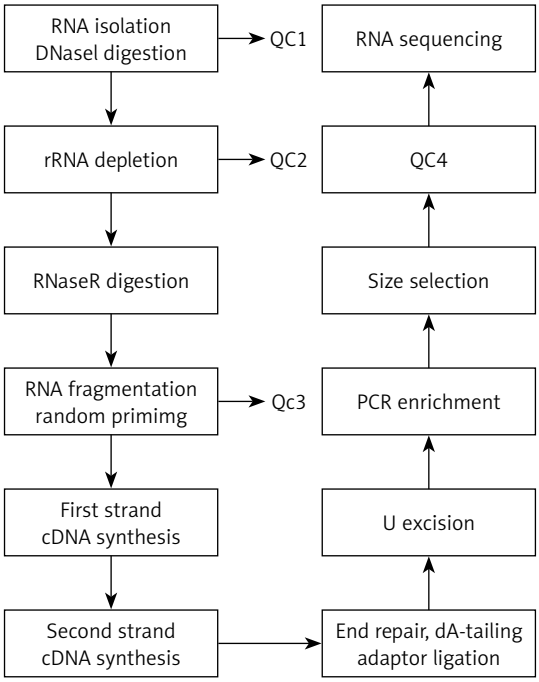

Figure 1. The main experimental process of circular RNA gene sequencing. QC1: detection of the total RNA concentration, purity, completeness; QC2: confirmed that more than 99% of the rRNA had been removed; QC3: confirmed that RNA was fragmented into approximately 200-bp fragments; QC4: detected the fragment concentration and size and library concentration. The distribution of the significantly differentially expressed miRNA of cells in the cellular component with high throughput sequencing

Isolation of RNA from exosomes

Exosome supernatants were added to 40 pM synthetic cel-miR-39 (UCACCGGGUGUAAAUCAGCUUG) to control and normalize the efficiency of RNA extraction; then, they were transferred to RNase-free tubes for RNA isolation using an miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The RNA sample was washed twice in 500 µl of RPE buffer and eluted in RNase-free water. The isolated RNA was measured using a NanoDrop 1000 ultraviolet spectrophotometer (Thermo Fisher Scientific) and analyzed by reverse transcription polymerase chain reaction (RT-PCR) followed by quantitative PCR (qPCR).

Serum and urine exosome circRNA sequencing

The total RNA was extracted and then was digested with DNase I to remove rRNA; then, RNase R was used to remove the linear RNA, enriching the circRNAs. The circRNAs were fragmented, and the first strand cDNA was synthesized by reverse transcription using random primers. Then, the second strand cDNA was synthesized using dNTP containing dUTP. The secondary chain product was subjected to terminal repair, and was pulsed "A" and a linker. The reaction mixture was digested with USER enzyme to remove the second strand cDNA containing dUTP, and a primer was added to amplify via PCR and obtain a chain-specific cDNA library. The fragments were screened by magnetic beads. Quality control was performed and fragments were further sequenced on a machine. The experimental procedure is summarized in Figure 1.

Bioinformatics analysis

The expression values calculated for the differential proteins and peptides were used for the distance and average to determine the linkage for gene ontology (GO) analysis. In pathway analysis, interactions between genes in the range of genomes were analyzed by downloading the pathway data in KEGG. Finally, the results of the above data were merged into a comprehensive gene inter-relationship network. The established gene network could directly reflect the inter-relationships between genes at a whole-cell level as well as the stability of the gene regulatory network.

Statistical analysis

The back-spliced junction reads and linear mapped reads were combined and scaled to reads

per million mapped reads (RPM) to quantify circRNA expression levels. Differences in circRNA expression levels were analyzed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Total RNA quality and concentration determination results

RNA was extracted and purified using an RNA isolation kit. The total RNA of the IMN and NC groups was detected with a Qubit3.0 fluorescence meter. The results are shown in Tables II and III. In the tables, the total amount of exosome RNA measured in each group was more than 200 ng, and the obtained circRNAs had high purity and good integrity, and could be used for later experiments.

Types of circRNAs

Compared with the healthy control group, the types of circRNAs in the serum of the patients with idiopathic membranous nephropathy decreased and mainly appeared as intron region sources. However, the circRNAs in the urinary exosomes increased, and mainly appeared to have an exon region source (Table IV).

Difference analysis of circRNAs

According to the expression level of circRNAs, when the difference multiple (ratio) was more than 2 or less than 0.5 and FDR ≤ 0.001, the circRNAs were considered differentiated. In this study, the log2 ratio was used instead of multiple differences. The filter criteria of significantly differentially expressed genes were FDR ≤ 0.001 and |log2 ratio| ≥ 1.

Differential expression of circRNAs in serum and urine exosomes of IMN patients

According to the experimental results, there were 59 species of circRNA with significantly different expression compared to serum and urine exosomes in IMN patients; 32 species were up-regulated (Table V) and 27 species were down-regulated (Table VI). Most of these circRNAs had an intron source. The corresponding genes were mainly SNORA25, SNORA31, SNORA51, SNORA75 and other nucleolus small RNAs. The log2 ratio of chrY: 13688616|13833086 was 27.592 in the up-regulation circRNA, which was the most significant. The log2 ratio of chrY:13842647|13855594 was –26.379 in the down-regulation circRNA, which was the most significant. However, the two most significantly different circRNAs in the circBase gene pool had no corresponding gene, suggesting that they may be newly discovered genes.

Differential expression of circRNAs in serum exosomes of IMN and NC patients

According to the experimental results, there were 89 species of circRNAs with significantly

Table II. Concentration of exosome total RNA

Sample name	Serum volume [ml]	Exosome RNA concentration [ng/μl]	Exosome RNA total amount [ng]
IMN group	28.7	9.69	242.30
NC group	23	33.40	400.80

IMN group – IMN group, NC group – normal control group.

Table III. Concentration of exosome total RNA

Sample name	Urine volume [ml]	Exosome RNA concentration [ng/μl]	Exosome RNA total amount [ng]
IMN group	980	16.50	445.50
NC group	970	16.70	367.40

IMN group – IMN group, NC group – normal control group.

Table IV. Species of exosome circRNA

Variable	IMN serum	IMN urine	NC serum	NC urine
Total number of circRNAs	85	286	227	12
Number of circRNAs from the circBase database	0	198	6	1
Number of circRNAs from the exon region	5	218	12	2
Number of circRNAs from the intron region	60	58	189	6
Number of circRNAs from the intergenic region	20	10	26	1

IMN group – IMN group, NC group – normal control group.

Table V. Up-regulated circRNAs between the IMN serum and IMN urine

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chrY:13688616|13833086	27.592	Up	Intergenic region	chrY	n/a
chrY:13650802|13725921	26.677	Up	Intergenic region	chrY	n/a
chrY:13844080|13851741	25.449	Up	Intergenic region	chrY	n/a
chr3:114874721|114874739	24.936	Up	Intron	chr3	SNORA25
chr4:49133318|49151812	24.363	Up	Intron	chr4	SNORA75
chr4:49133318|49151817	23.766	Up	Intron	chr4	SNORA75
chr1:246981249|246981308	23.51	Up	Intron	chr1	SNORA25
chr6:39390231|39390251	23.444	Up	Intron	chr6	KIF6
chrY:13688616|13810318	23.251	Up	Intergenic region	chrY	n/a
chrY:13867301|13869486	23.116	Up	Intergenic region	chrY	n/a
chr3:42154842|42154889	23.059	Up	Intron	chr3	TRAK1
chr8:70602312|70602409	22.531	Up	Intron	chr8	SLCO5A1
chr4:49118019|49128722	22.398	Up	Intron	chr4	SNORA51
chr10:39103465|39105726	22.302	Up	Intron	chr10	SNORA31
chr8:70602353|70602431	22.029	Up	Intron	chr8	SLCO5A1
chr8:70602360|70602427	21.967	Up	Intron	chr8	SLCO5A1
chr10:39085864|39088295	21.903	Up	Intron	chr10	SNORA31
chr1:91853081|91853139	21.766	Up	Intron	chr1	SNORA31
chrY:13684026|13844079	21.614	Up	Intergenic region	chrY	n/a
chr3:114874721|114874743	21.614	Up	Intron	chr3	SNORA25
chr8:70602355|70602427	21.531	Up	Intron	chr8	SLCO5A1
chr4:49637530|49641867	21.351	Up	Intron	chr4	SNORA51
chr4:49120156|49121084	21.351	Up	Intron	chr4	SNORA51
chr8:70602312|70602382	21.351	Up	Intron	chr8	SLCO5A1
chr17:22246001|22253301	21.351	Up	Intron	chr17	snoU13
chr21:10778969|10808326	21.251	Up	Intron	chr21	SNORA70
chr1:108113527|108113595	21.251	Up	Intron	chr1	SNORA31
chrY:13659053|13844079	21.144	Up	Intron	chrY	n/a
chr15:101250552|101250653	21.144	Up	Intron	chr15	snoU13
chr21:44593818|44593903	21.144	Up	Intergenic region	chr21	n/a
chr7:100550808|100551062	4.267	Up	Exon	chr7	MUC3A
chrY:13805036|13841134	3.876	Up	Intergenic region	chrY	n/a

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

different expression compared to IMN patients' serum exosomes and NC patients' serum exosomes; 49 species were up-regulated (Table VII) and 40 species were down-regulated (Table VIII). Most of these circRNAs had an intron source. The corresponding genes were mainly SNORA25, SNORA51, SNORA31, SNORA75, SNORD112 and other nucleolus small RNAs. The log2 ratio of chrY:13688616|13833086 was 27.592 in the up-regulation circRNAs, which was the most significant. However, the circRNAs in the circBase gene pool had no corresponding gene, which sug-

Table VI. Down-regulated circRNAs between the IMN serum and IMN urine

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chrY:13842647|13855594	–26.379	Down	Intergenic region	chrY	n/a
chrY:13650802|13659298	–26.116	Down	Intergenic region	chrY	n/a
chr17:22248380|22253301	–25.516	Down	Intron	chr17	snoU13
chr8:43092760|43093139	–25.146	Down	Intron	chr8	SNORD112
chr8:43092873|43096758	–24.588	Down	Intron	chr8	SNORD112
chr4:90986390|90986415	–24.441	Down	Intron	chr4	SNORA51
chr4:49103783|49111822	–23.791	Down	Intron	chr4	SNORA51
chr10:39139428|39147131	–23.244	Down	Intron	chr10	SNORA31
chr4:49641376|49652154	–23.221	Down	Intron	chr4	SNORA51
chr18:54265993|54266355	–23.079	Down	Exon	chr18	TXNL1
chr6:158779108|158779264	–22.894	Down	Intron	chr6	TULP4
chr2:19441309|19442090	–22.806	Down	Intron	chr2	SNORA51
chr3:96221435|96221837	–22.776	Down	Intron	chr3	SNORA25
chrY:13801063|13849765	–22.266	Down	Intergenic region	chrY	n/a
chr8:43095798|43096720	–21.976	Down	Intron	chr8	SNORD112
chr6:61899754|61913064	–21.806	Down	Intron	chr6	SNORD45
chr8:43093689|43097076	–21.681	Down	Intron	chr8	SNORD112
chr2:233244474|233272478	–21.614	Down	Intron	chr2	snoU13
chr2:221311242|221311332	–21.543	Down	Intron	chr2	SNORA75
chr20:30954187|30956926	–21.543	Down	Exon	chr20	ASXL1
chr4:35172567|35172590	–21.469	Down	Intron	chr4	SNORA75
chr6:2024936|2340390	–21.391	Down	Intron	chr6	snoU13
chr10:18831781|18831900	–21.309	Down	Intron	chr10	SNORA31
chr10:42400571|42533897	–21.221	Down	Intron	chr10	SNORA31
chr15:30465080|30465505	–21.128	Down	Intron	chr15	SNORA48
chr19:34882415|34883413	–3.063	Down	Intron	chr19	GPI
chrY:13691698|13851741	–1.454	Down	Intergenic region	chrY	n/a

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

gested that it may be a newly discovered gene. The log2 ratio of chr2:233244474|233272478 was –27.111 in the down-regulation circRNAs, which was the most significant, and the corresponding gene is the snoU13 gene. This gene is mainly expressed nucleolus small RNA and plays a role in RNA treatment and modification.

Differential expression of circRNAs in urine exosomes of IMN and NC patients

According to the experimental results, there were 60 species of circRNAs with significantly different expression compared to IMN patients' urine exosomes and NC patients' urine exosomes; 54 species were up-regulated (Table IX) and 6 species were down-regulated (Table X). Approximately 55% were intron sources, 30% were exon sources and 15% were intergenic regions. The corresponding genes were mainly SNORA51, SNORA31, SNORA70, SNORA75, SNORD112 and other nucleolus small RNAs. The log2 ratio of chrY:13842647|13855594 was 26.379 in the up-regulated circRNA, which was the most significant. The log2 ratio of chrY:13688616|13833086 was –25.049 in the down-regulated circRNAs, which was the most significant. However, the two

Table VII. Up-regulated circRNAs between the IMN and NC groups in serum

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chrY:13688616|13833086	27.592	Up	Intergenic region	chrY	n/a
chrY:13650802|13725921	26.677	Up	Intergenic region	chrY	n/a
chr3:114874721|114874739	24.936	Up	Intron	chr3	SNORA25
chr4:49133318|49151812	24.363	Up	Intron	chr4	SNORA51
chr4:49133318|49151817	23.766	Up	Intron	chr4	SNORA51
chr6:39390231|39390251	23.444	Up	Intron	chr6	KIF6
chrY:13688616|13810318	23.251	Up	Intergenic region	chrY	n/a
chr3:42154842|42154889	23.059	Up	Intron	chr4	TRAK1
chr4:49118019|49128722	22.398	Up	Intron	chr4	SNORA51
chr10:39103465|39105726	22.302	Up	Intron	chr10	SNORA31
chr10:39085864|39088295	21.903	Up	Intron	chr10	SNORA31
chr1:91853081|91853139	21.766	Up	Intron	chr1	HFM1
chrY:13684026|13844079	21.614	Up	Intergenic region	chrY	n/a
chr3:114874721|114874743	21.614	Up	Intron	chr3	SNORA25
chr8:70602355|70602427	21.531	Up	Intron	chr8	SLCO5A1
chr4:49637530|49641867	21.351	Up	Intron	chr4	SNORA51
chr4:49120156|49121084	21.351	Up	Intron	chr4	SNORA51
chr17:22246001|22253301	21.351	Up	Intron	chr17	snoU13
chr21:10778969|10808326	21.251	Up	Intron	chr21	SNORA70
chr1:108113527|108113595	21.251	Up	Intron	chr1	SNORA51
chrY:13659053|13844079	21.144	Up	Intergenic region	chrY	n/a
chr15:101250552|101250653	21.144	Up	Intron	chr15	snoU13
chr21:44593818|44593903	21.144	Up	Intergenic region	chr21	n/a
chr2:5845511|5845954	20.766	Up	Intron	chr2	snoU13
chr4:70296654|70296710	20.614	Up	Intron	chr4	SNORA51
chr7:71387989|71388027	20.614	Up	Intron	chr7	CALN1
chr16:47538682|47538754	20.614	Up	Intron	chr16	PHKB
chr19:34882415|34883413	20.444	Up	Intron	chr19	GPI
chr2:92305623|92309358	20.444	Up	Intron	chr2	SNORA75
chr20:59906635|59906776	20.251	Up	Intron	chr20	CDH4
chr1:91852914|91852996	20.029	Up	Intron	chr1	HFM1
chr10:38778641|38816581	20.029	Up	Intron	chr10	SNORA31
chrX:108297654|108297709	20.029	Up	Exon	chrX	CTD-2328D6.1
chr12:38237430|38502951	20.029	Up	Intron	chr12	SNORD112
chr20:59906715|59906776	19.766	Up	Intron	chr20	CDH4
chr14:70396886|70396954	19.766	Up	Intron	chr14	SMOC1
chr8:70602368|70602431	19.766	Up	Intron	chr8	SLCO5A1
chr10:51358680|51636067	19.766	Up	Intron	chr10	SNORA31
chr8:70602312|70602420	19.766	Up	Intron	chr8	SLCO5A1
chr8:70602360|70602427	4.413	Up	Intron	chr8	SLCO5A1
chr1:246981249|246981308	3.955	Up	Intron	chr1	SNORA25
chr7:100550808|100551062	3.349	Up	Exon	chr7	MUC3A
chrY:13867301|13869486	2.807	Up	Intergenic region	chrY	n/a
chrY:13805036|13841134	2.806	Up	Intergenic region	chrY	n/a
chr8:70602353|70602431	2.567	Up	Intron	chr8	SLCO5A1
chrY:13691698|13851741	2.522	Up	Intergenic region	chrY	n/a
chr8:70602312|70602409	2.276	Up	Intron	chr8	SLCO5A1
chrY:13844080|13851741	2.012	Up	Intergenic region	chrY	n/a
chrY:13688616|13851691	1.936	Up	Intergenic region	chrY	n/a

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

Table VIII. Down-regulated circRNA between the IMN and NC groups in serum

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chr2:233244474|233272478	–27.111	Down	Intron	chr2	snoU13
chr17:39537965|39552828	–26.966	Down	Intron	chr17	SCARNA20
chr22:42910112|42970824	–26.895	Down	Intron	chr22	Y_RNA
chr6:31122297|31122344	–26.054	Down	Exon	chr6	CCHCR1
chr12:52863454|52909616	–25.798	Down	Intron	chr12	SNORD112
chr19:36066505|36066634	–25.772	Down	Intron	chr19	SNORA70
chr4:1005136|1242947	–25.454	Down	Intergenic region	chr4	n/a
chr4:159973545|159973572	–25.028	Down	Intron	chr4	SNORA51
chrY:13842647|13855594	–24.668	Down	Intergenic region	chrY	n/a
chr21:37558665|37558690	–22.588	Down	Intron	chr21	DOPEY2
chr15:31645251|31645272	–22.461	Down	Intron	chr15	KLF13
chr10:39139428|39141998	–22.387	Down	Intron	chr10	SNORA31
chr17:39938846|39938869	–22.336	Down	Intron	chr17	JUP
chr17:22253135|22260437	–22.198	Down	Intron	chr17	snoU13
chr17:79502678|79502749	–21.981	Down	Intron	chr17	FSCN2
chr17:48266264|48272839	–21.912	Down	Exon	chr17	COL1A1
chr10:38804894|38818467	–21.894	Down	Intron	chr10	SNORA31
chr1:74953936|74953971	–21.858	Down	Intron	chr1	TMEM56
chr5:116075463|116075487	–21.764	Down	Intron	chr5	SNORA70
chr18:18518121|18519655	–21.764	Down	Intron	chr18	SNORD112
chr7:148028455|148028529	–21.744	Down	Intron	chr7	CNTNAP2
chr2:189121958|189121979	–21.724	Down	Intron	chr2	SNORA48
chrX:3349826|3349848	–21.642	Down	Intron	chrX	snoU13
chr11:75979847|75979884	–21.599	Down	Intron	chr11	SNORA1
chr15:42134880|42134903	–21.509	Down	Exon	chr15	PLA2G4B
chr18:32291302|32291329	–21.387	Down	Intron	chr18	DTNA
chr13:36337738|36337787	–21.362	Down	Intron	chr13	SNORA25
chr17:31559413|31559527	–21.336	Down	Intron	chr17	ASIC2
chr9:19592476|19592555	–21.282	Down	Intron	chr9	SLC24A2
chr7:76626497|76626556	–21.282	Down	Intron	chr7	DTX2P1
chr8:124924619|124924638	–21.198	Down	Intron	chr8	FER1L6
chr1:7769121|7769144	–21.078	Down	Intron	chr1	CAMTA1
chr14:37211610|37211628	–21.046	Down	Intron	chr14	SLC25A21
chr1:32294226|32294254	–20.947	Down	Intron	chr1	SNORA70
chr17:31559408|31559527	–20.912	Down	Intron	chr17	ASIC2
chr19:56438931|56438947	–20.84	Down	Intron	chr19	NLRP13
chr1:155048684|155048737	–20.764	Down	Intron	chr1	EFNA3
chr20:46681136|46681159	–20.764	Down	Intron	chr20	snoU13
chr1:233454768|233454781	–20.764	Down	Intron	chr1	SNORA25
chr6:104238460|104238484	–3.592	Down	Intron	chr6	SNORA33

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

Table IX. Up-regulated circRNAs between the IMN and NC groups in urine

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chrY:13842647|13855594	26.379	Up	Intergenic region	chrY	n/a
chrY:13691698|13851741	26.006	Up	Intergenic region	chrY	n/a
chr17:22248380|22253301	25.516	Up	Intron	chr17	snoU13
chr8:43092760|43093139	25.146	Up	Intron	chr8	SNORD112
chr8:43092873|43096758	24.588	Up	Intron	chr8	SNORD112
chr4:49103783|49111822	23.791	Up	Intron	chr4	SNORA51
chr19:34882415|34883413	23.507	Up	Intron	chr19	GPI
chr10:39139428|39147131	23.244	Up	Intron	chr10	SNORA31
chr4:49641376|49652154	23.221	Up	Intron	chr4	SNORA51
chr18:54265993|54266355	23.079	Up	Exon	chr18	TXNL1
chr6:158779108|158779264	22.894	Up	Intron	chr6	TULP4
chr2:19441309|19442090	22.806	Up	Intron	chr2	SNORA51
chr3:96221435|96221837	22.776	Up	Intron	chr3	SNORA25
chrY:13801063|13849765	22.266	Up	Intergenic region	chrY	n/a
chr8:43095798|43096720	21.976	Up	Intron	chr8	SNORD112
chr6:61899754|61913064	21.806	Up	Intron	chr6	SNORD45
chr8:43093689|43097076	21.681	Up	Intron	chr8	SNORD112
chr2:233244474|233272478	21.614	Up	Intron	chr2	snoU13
chr2:221311242|221311332	21.543	Up	Intron	chr2	SNORA75
chr20:30954187|30956926	21.543	Up	Exon	chr20	ASXL1
chr4:35172567|35172590	21.469	Up	Intron	chr4	SNORA75
chr6:2024936|2340390	21.391	Up	Intron	chr6	snoU13
chr10:18831781|18831900	21.309	Up	Intron	chr10	SNORA31
chr10:42400571|42533897	21.221	Up	Intron	chr10	SNORA31
chr15:30465080|30465505	21.128	Up	Intron	chr15	SNORA48
chr21:10788458|10853762	21.029	Up	Intron	chr21	SNORA70
chr18:18518121|18519655	20.806	Up	Intron	chr18	SNORD112
chrY:13805036|13841134	20.806	Up	Intergenic region	chrY	n/a
chr3:196118684|196129890	20.681	Up	Exon	chr3	UBXN7
chr5:137320946|137324004	20.543	Up	Exon	chr5	FAM13B
chr9:137976113|137976207	20.543	Up	Intron	chr9	OLFM1
chr4:49101961|49155306	20.543	Up	Intron	chr4	SNORA75
chr11:33307959|33309057	20.391	Up	Exon	chr11	HIPK3
chr17:20107646|20109225	20.221	Up	Exon	chr17	SPECC1
chr19:7034465|7036161	20.221	Up	Intron	chr19	Y_RNA
chr8:141874411|141900868	20.029	Up	Exon	chr8	PTK2
chr8:99718695|99719539	20.029	Up	Exon	chr8	STK3
chr13:64330137|64398060	20.029	Up	Intron	chr13	SNORA25
chr8:43093228|43097076	20.029	Up	Intron	chr8	SNORD112
chrY:13140123|13456953	20.029	Up	Intergenic region	chrY	n/a
chr21:16386665|16415895	19.806	Up	Exon	chr21	NRIP1
chr10:32197100|32199491	19.806	Up	Exon	chr10	ARHGAP12
chr14:76633006|76662315	19.806	Up	Exon	chr14	GPATCH2L
chrY:13137990|13450019	19.806	Up	Intergenic region	chrY	n/a
chr21:10789780|10836717	19.543	Up	Intron	chr21	SNORA70
chr2:61749746|61761038	19.543	Up	Exon	chr2	XPO1
chr5:72370569|72373320	19.543	Up	Exon	chr5	FCHO2
chr1:180953813|180962561	19.543	Up	Exon	chr1	STX6
chr2:228252617|228252643	19.543	Up	Intron	chr2	SNORA75
chr2:202010101|202014558	19.543	Up	Exon	chr2	CFLAR
chr20:52773708|52788209	19.543	Up	Exon	chr20	CYP24A1
chr9:113734353|113735838	19.543	Up	Exon	chr9	LPAR1
chr6:4891947|4892613	4.346	Up	Exon	chr6	CDYL
chrY:13650802|13659298	1.893	Up	Intergenic region	chrY	n/a

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

Table X. Down-regulated circRNAs between the IMN and NC groups in urine

circRNA	Log2 ratio	Up/down	CircRNA type	Chromosome localization	Gene localization
chrY:13688616|13833086	–25.049	Down	Intergenic region	chrY	n/a
chr17:25267933|25267961	–23.323	Down	Intron	chr17	snoU13
chr10:39084961|39105726	–23.142	Down	Intron	chr10	SNORA31
chr10:38787997|39138199	–22.485	Down	Intron	chr10	SNORA31
chr14:105944010|105944069	–20.583	Down	Intron	chr14	CRIP2
chrY:13688616|13851691	–1.126	Down	Intergenic region	chrY	n/a

NB: Gene ID n/a indicates that there was no matched circRNA in the circBase gene bank.

most significantly different circRNAs in the circBase gene pool had no corresponding gene, suggesting that they may be newly discovered genes.

Bioinformatics analysis

Target genes were analyzed for their potential functions using GO and KEGG pathways. GO analysis demonstrated that the target genes were associated with cellular processes, multicellular organisms, pigmentation, the development process and the response to stimuli at both serum and urine exosomes (Figures 2 and 3). Furthermore, significantly associated pathways comprising the target genes were obtained for the assessed circRNAs. Interestingly, we selected 29 metabolic pathways in the serum sample; of all 29 pathways, 21 had PLA abnormalities, and the corresponding gene was PLA2G4B. The top 20 signaling pathways are shown in Figure 4, while the platelet activation signaling pathway was the most widely distributed (Figure 5). In addition, we selected 35 metabolic pathways in the urine samples. The top 20 are shown in Figure 6, while the P13K-Akt signaling pathway was the most widely distributed (Figure 7).

Discussion

Beck *et al.* [24] detected anti-PLA2R antibodies for the first time in IMN patient plasma samples. Substantial clinical data showed its specificity of up to 100% and sensitivity of approximately 70% to 80%, which indicated that they can be used as IMN-specific diagnostic markers.

The latest study [24, 25] showed that the mannose-binding lectin pathway was the major complement activation in the pathogenesis of IMN. In this study, the mucin 3A (MUC3A) gene, corresponding to the circRNAs of chromosome 7 encoding chr7: 100550808|100551062 in the serum exosomes of IMN patients, was significantly

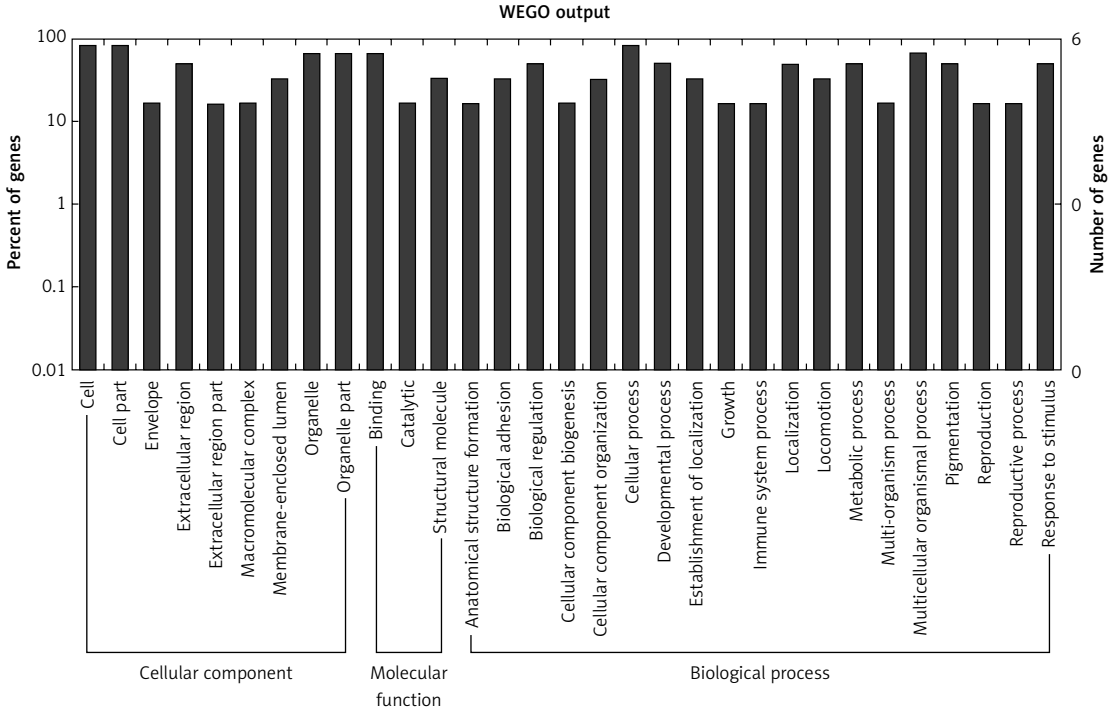

Figure 2. GO annotation of differentially expressed circRNAs in the serum exosomes of IMN patients compared to the control group. GO annotation consisted of the biological process, cellular components, and molecular function

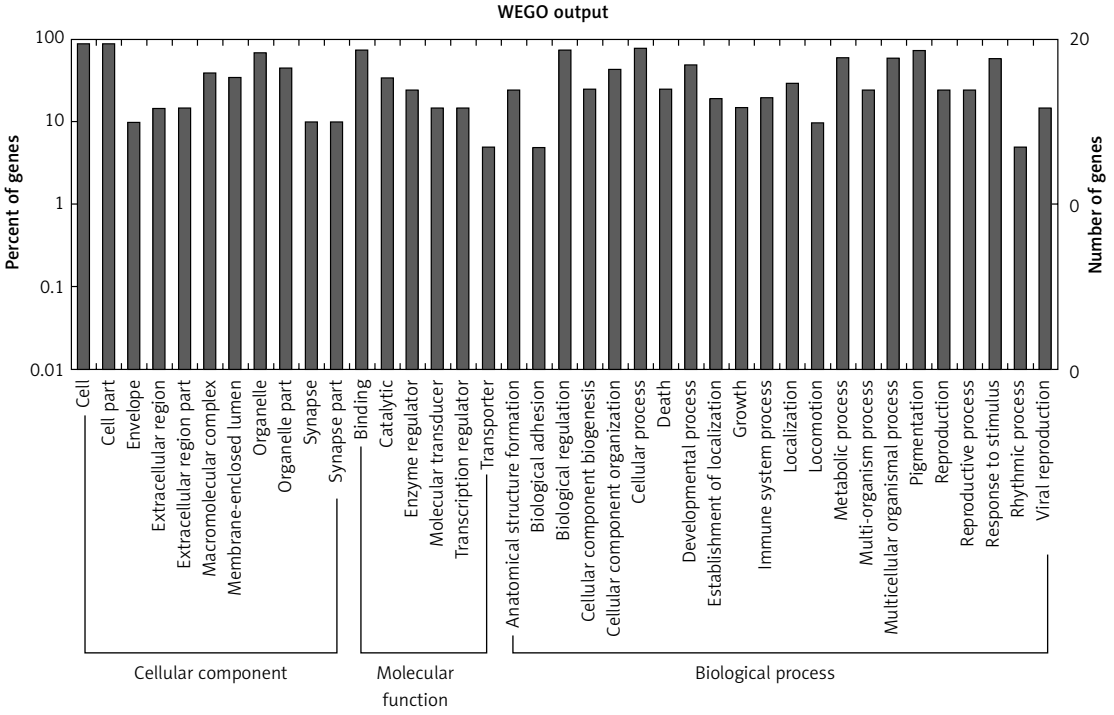

Figure 3. GO annotation of differentially expressed circRNAs in urine exosomes of IMN patients compared to the control group. GO annotation consisted of the biological process, cellular component, and molecular function

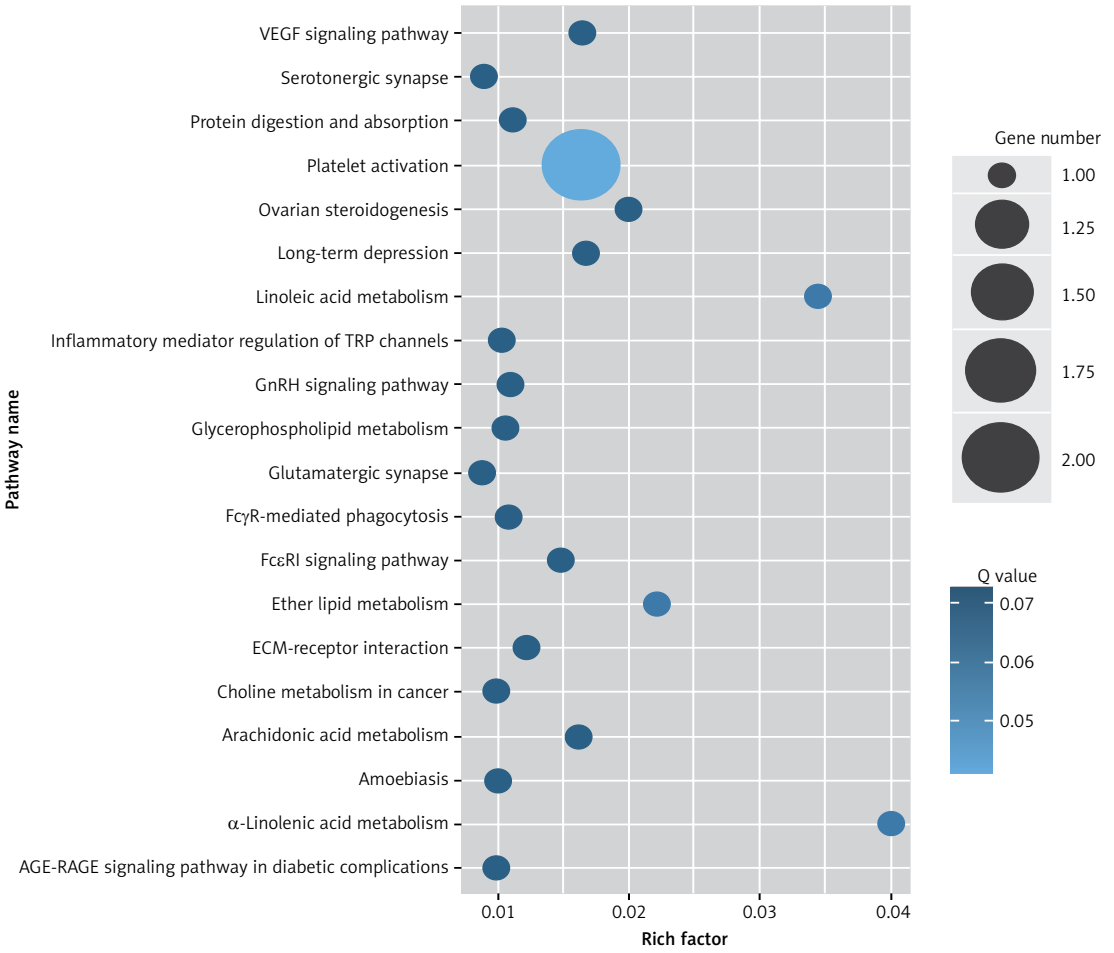

Figure 4. KEGG pathway analysis of predicted targets for differentially expressed circRNAs in serum exosomes of IMN patients compared to the control group. The bluer the circle, the more significant the pathway enrichment. The bigger the circle, the higher the number of pathway genes

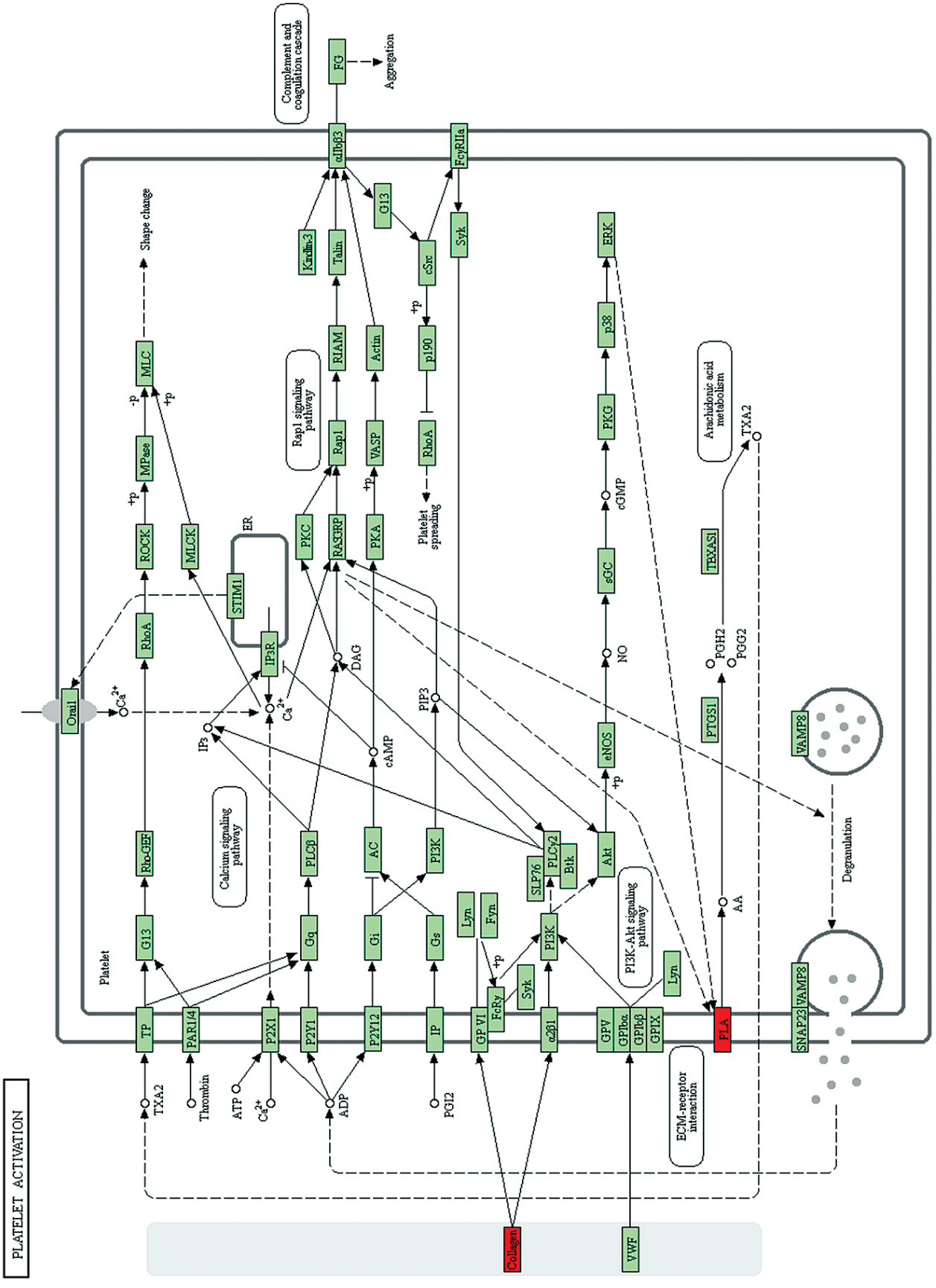

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Figure 5. Pathway analysis of differential genes: platelet activation. Red marks indicate the genes with differential profiles

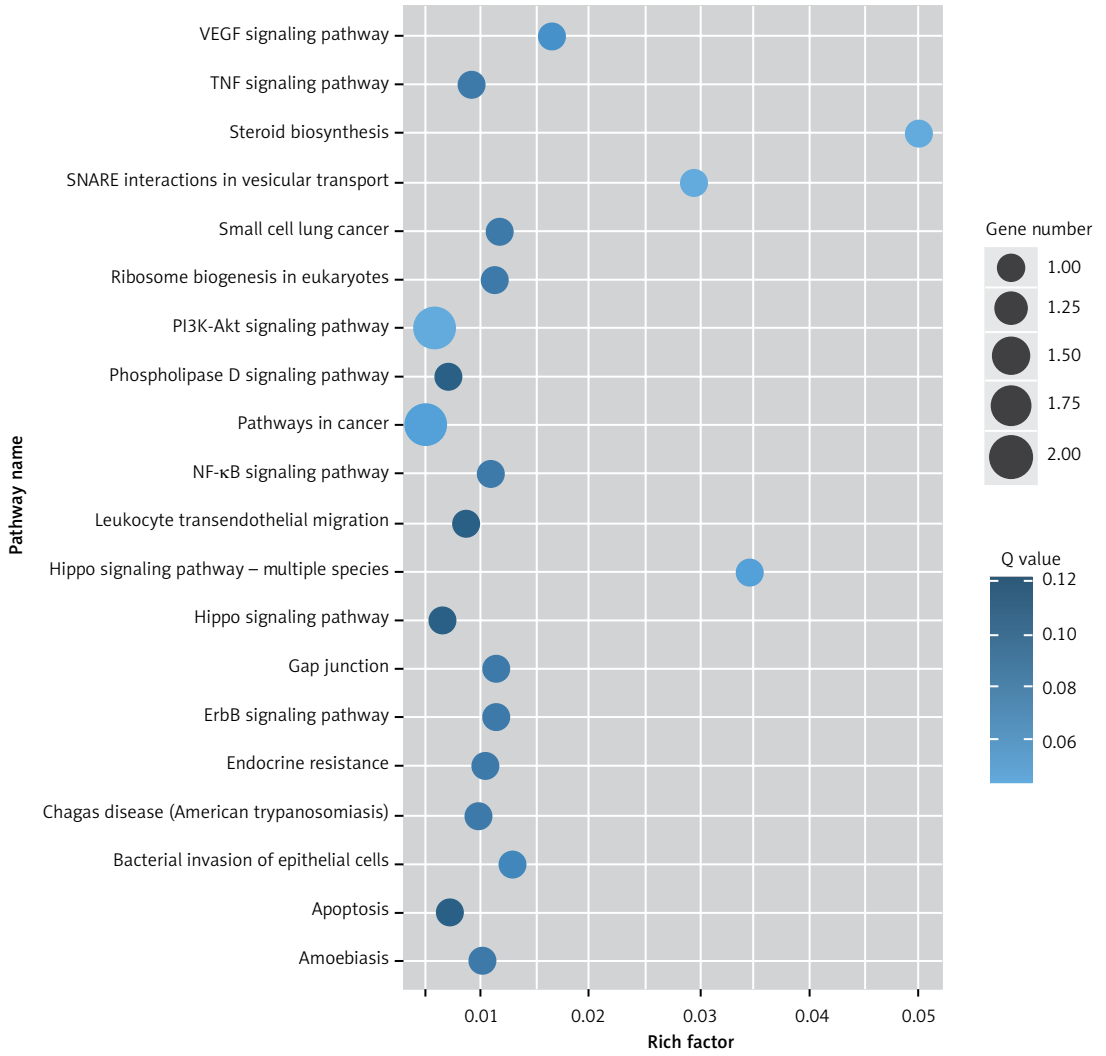

Figure 6. KEGG pathway analysis of predicted targets for differentially expressed circRNAs in urine exosomes of IMN patients compared to the control group. The bluer the circle, the more significant the pathway enrichment. The bigger the circle, the higher the number of pathway genes

up-regulated. It was also found that MUC3A was encoded by an exon-derived gene. Existing studies have shown that [26] MUC3A is a mucin cluster located on the 7p22 chromosome. Additionally, MUC3A belongs to a transmembrane glycoprotein. Authors [27] found that 71% of the amino acid repeated sequences encoded by MUC3 were serine/threonine and 6% proline. Studies have demonstrated that the activation of serine proteases is achieved by a change in specific amino acid residues in the center of serine-dominated activity [28]. Because most of the amino acids encoded by the MUC3A gene in this study were serine/threonine, we speculate that the MUC3A gene may encode the relevant amino acids and then play an important role in the pathogenesis of IMN through the mannose-binding lectin pathway. Previous evidence suggests that PLA2R-IgG4 can play a role by activating the complement lectin pathway with MBL [29]. The serine of the MUC3A gene also plays a role in the lectin binding pathway. Therefore, we further speculated that the MUC3A gene may be associated with IgG4 and anti-PLA2R antibody expression. There were some relationships in the diagnosis and prognosis of IMN. In addition, it was reported [30] that MUC3A is a class of membrane-associated mucins, which can mediate some of the particles and related pathogens adhering to the mucosal surface. Additionally, MUC3A is involved in binding of the receptor and ligand and signal transduction pathways. MUC3A can mediate the adhesion of the relevant particles to the membrane surface and participate in the receptor ligand binding process, suggesting that MUC3A may also play a role in the formation of immune complexes.

In addition, in this experiment, the genes for which we observed a significant difference in the circRNAs are mainly intron-derived circRNAs. The corresponding genes are SNORA25, SNORA31,

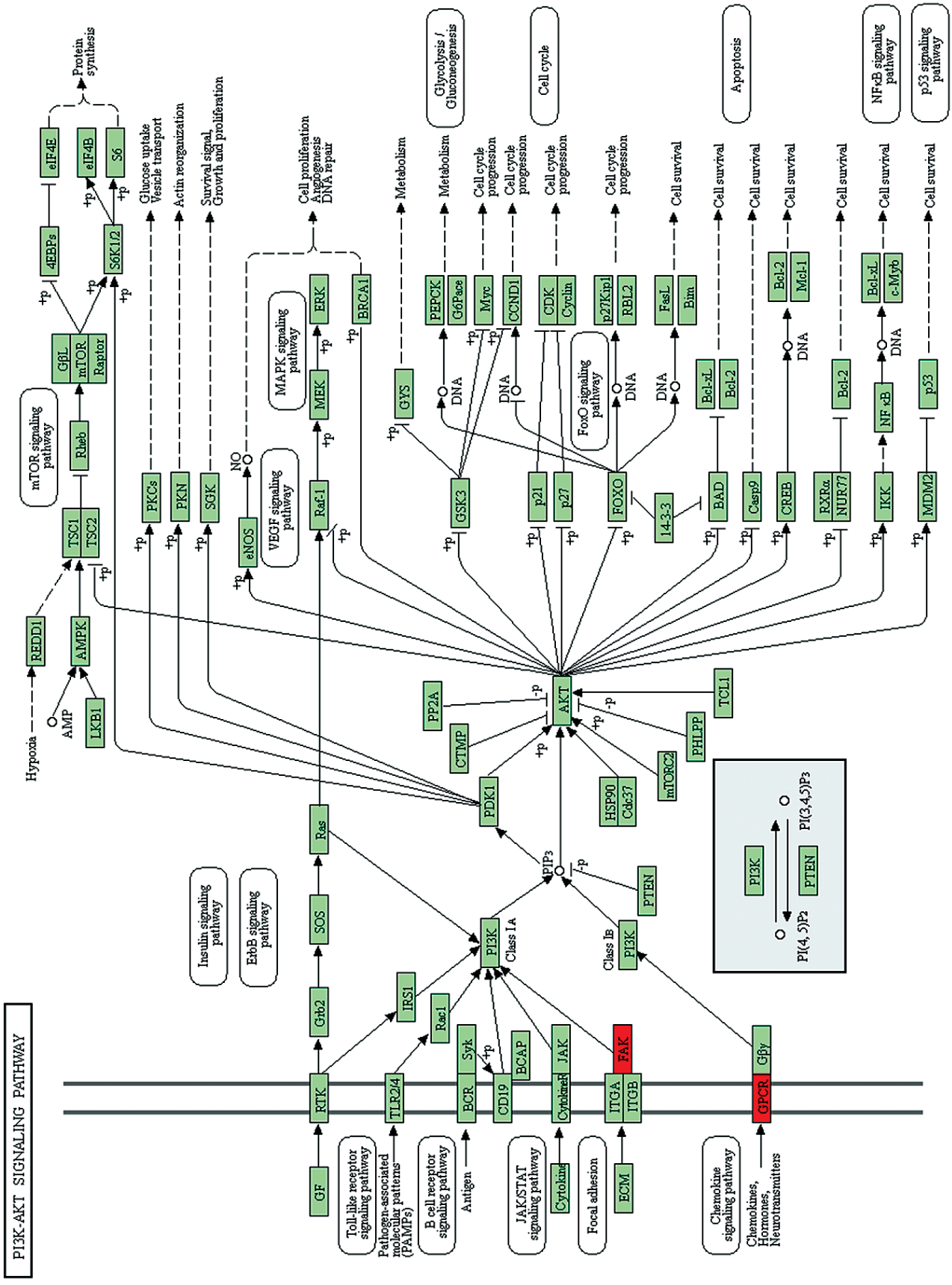

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Figure 7. Pathway analysis of differential genes: the PI3K-Akt signaling pathway. Red marks indicate the genes with differential profiles

SNORA70, SNORA75, SNORD112 and other small nucleolar RNAs (snoRNAs). An increasing number of studies have shown that snoRNAs can be further processed to form shorter RNA fragments, and these short fragments of snoRNAs have microRNA-like functions. This finding suggested that snoRNAs may act as microRNA precursors [31]. One study [32, 33] showed that circRNAs of different gene sources exist in different parts of the cell and the function is also different. The corresponding genes of circRNAs that we obtained in this experiment were mainly the intron source for coding snoRNAs. Therefore, we speculate that in the pathogenesis of IMN at the gene level, the circRNAs of the intron source may code snoRNAs that modify the mRNA during and before transcription as well as regulating the gene expression at the mRNA level.

Studies have shown that alleles-PLA2R1 and HLA-DQA1 are closely related to IMN [34]. In this study, we selected 29 metabolic pathways in the serum sample; 21 had PLA abnormalities. IMN does not appear to occur through a specific signaling pathway; instead, several pathways appear to work at the same time. Additionally, the corresponding gene of PLA was PLA2G4B, which corresponds to PLA2R positivity in IMN patients. IMN may be associated with the PLA2G4B gene. Therefore, evaluation of PLA2G4B may provide new clues for the diagnosis and treatment of IMN.

In conclusion, we found that there were abnormal expression levels of circRNAs in serum and urine exosomes in IMN patients. These circRNAs with abnormal expression could be involved in IMN pathogenesis. However, the specific mechanism and function of the circRNAs with differential expression in the disease require more direct evidence. However, with the continuous development of biological technology and continuous research on circRNAs, circRNAs will eventually provide a new theoretical basis in the disease diagnosis, treatment and prognosis. Additionally, the study of PLA2G4B may provide new clues for the diagnosis and treatment of IMN.

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Hualin Ma and Ying Xu contributed equally to the work.

Conflict of interest

The authors declare no conflict of interest.

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