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Both Peripheral Blood and Urinary miR-195-5p, miR-192-3p, miR-328-5p and Their Target Genes PPM1A, RAB1A and BRSK1 May Be Potential Biomarkers for Membranous Nephropathy

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Data Interpretation D
Manuscript Preparation E
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Background: To identify noninvasive diagnostic biomarkers for membranous nephropathy (MN).

Material/Methods: The mRNA microarray datasets GSE73953 using peripheral blood mononuclear cells (PBMCs) of 8 membranous nephropathy patients and 2 control patients; and microRNAs (miRNA) microarray dataset GSE64306 using urine sediments of 4 membranous nephropathy patients and 6 control patients were downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) were respectively identified from PBMCs and urine sediments of membranous nephropathy patients, followed with functional enrichment analysis, protein-protein interaction (PPI) analysis, and miRNA-target gene analysis. Finally, the DEGs and the target genes of DEMs were overlapped to obtain crucial miRNA-mRNA interaction pairs for membranous nephropathy.

Results: A total of 1246 DEGs were identified from PBMCs samples, among them upregulated CCL5 was found to be involved in the chemokine signaling pathway, and BAX was found to be apoptosis related; while downregulated PPM1A and CDK1 were associated with the MAPK signaling pathway and the p53 signaling pathway, respectively. The hub role of CDK1 (degree=18) and CCL5 (degree=12) were confirmed after protein-protein interaction network analysis in which CKD1 could interact with RAB1A. A total of 28 DEMs were identified in urine sediments. The 276 target genes of DEMs were involved in cell cycle arrest (PPM1A) and intracellular signal transduction (BRSK1). Thirteen genes were shared between the DEGs in PMBCs and the target genes of DEMs in urine sediments, but only hsa-miR-192-3p-RAB1A, hsa-miR-195-5p-PPM1A, and hsa-miR-328-5p-BRSK1 were negatively related in their expression level.

Conclusions: Both peripheral blood and urinary miR-195-5p, miR-192-3p, miR-328-5p, and their target genes PPM1A, RAB1A, and BRSK1 may be potential biomarkers for membranous nephropathy by participating in inflammation and apoptosis.

MeSH Keywords: Apoptosis • Biological Markers • Glomerulonephritis, Membranous • Inflammation Mediators • MicroRNAs

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Background

Membranous nephropathy (MN) is one of the commonly diagnosed kidney diseases, with the incidence rate increased by more than 2 times from 2003 to 2014 in China [1]. It is estimated that approximately 20% of patients with MN will progress to end stage renal disease and 10% of them will die within 5 to 10 years [2,3]. Thus, accurate and timely diagnosis is required to prevent possible progression and improve patient outcomes.

Currently, renal biopsy and histological evaluation remain as the standard procedure for diagnosis of MN. However, renal biopsy is invasive, which may cause some unanticipated complications in the patients at high risk [4,5]. Hence, there is an urgent need to identify some noninvasive diagnostic biomarkers for MN.

The majority of MN is idiopathic (IMN) and the remainder is secondary to systemic autoimmune disease, certain malignancies, infections, or exposure to environmental factors [6]. Previous studies have shown that circulating autoantibodies against the M-type phospholipase A2 receptor (anti-PLA2R) are detectable in 80% of IMN patients and thus serum anti-PLA2R has been widely accepted as a biomarker for clinical diagnosis of IMN [7]. The coincidence rate of serum anti-PLA2R antibody and PLA2R in kidney tissue has been demonstrated to be 100% [8]. The remainder of MN cases may be associated with the inflammatory state [9] and therefore several circulatory immune cells and inflammatory cytokines have also been suggested as biomarkers for secondary MN. For example, Zhang et al. detected that concentrations of serum interleukin (IL)-2, IL-4, IL-10, IL-17A, and interferon (IFN)-γ were higher in the MN patients compared to controls [10]. Liu et al. observed the percentages of CD38(+) CD19(+), IL-10(+) CD19(+) B cells, and serum IL-10 level in MN patients were significantly higher than in health controls. The percentage of CD38(+) CD19(+) was positively correlated with the 24-hour urine protein concentration and negatively correlated with the value of estimated glomerular filtration rate (eGFR), which was the opposite with IL-10(+) CD19(+) B cells and serum IL-10 level [11]. Nagasawa et al. [12] used DNA microarray analysis to search peripheral blood mononuclear cells (PBMCs) mRNA for genes that had expression levels that distinguished between patients with MN and healthy volunteers. They proposed that IFN-alpha-inducible protein 27 in the peripheral blood could serve as a noninvasive marker for MN diagnosis. However, the clinical applications remain rare and further studies are still needed to investigate novel biomarkers for MN.

MicroRNAs (miRNAs) are a class of small noncoding RNA that negatively regulate gene expression by translational repression or induction of messenger RNA degradation. Recently, miRNAs have been found to be implicated in the development of nephropathy by influencing the inflammatory related genes [13,14] and thus they may also be underlying biomarkers for MN. This hypothesis was demonstrated in the study of Li et al. that found miR-217 regulated the expression of TNF superfamily member 11 (TNFSF11). Compared with control patients, miR-217 was significantly downregulated and TNFSF11 was significantly upregulated in MN patients. The patients with MN and the control patients could then be obviously separated according to the expression of miR-217, with an area under the curve of 0.941, and sensitivity and specificity of 88.9% and 75.9% respectively [13]. However, miRNA biomarkers for diagnosis of MN remain rarely reported [15].

The goal of this study was to further screen inflammatory related miRNA-mRNA biomarkers in both blood and urine samples for MN by using the microarray datasets downloaded from a public database. The miRNA-mRNA interaction and their function were also investigated. Our study may provide more noninvasive diagnostic biomarkers for MN.

Material and Methods

Data collection

The mRNA microarray data under accession number GSE73953 contributed by Nagasawa et al. [12] was downloaded from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) (*www.ncbi.nlm.nih.gov/geo*) based on the platform of GPL4133 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F. This dataset consisted of 25 PBMCs samples from 8 MN patients and 2 pooled healthy participants.

The miRNA expression profile under accession number GSE64306 deposited by Wang et al. [16] was also downloaded from GEO on the basis of the platform of GPL19117 [miRNA-4] Affymetrix Multispecies miRNA-4 Array. The miRNA expression analysis was performed in urine sediments from 4 MN patients and 6 healthy participants.

Data preprocessing and differential expression analysis

The raw CEL files were preprocessed and normalized using the Robust Multichip Average (RMA) algorithm [17] in the R Bioconductor affy package (version 3.4.1; *http://www.bioconductor.org/packages/release/bioc/html/affy.html*). The differentially expressed genes (DEGs) and the differentially expressed miRNAs (DEMs) between MN patients and controls were screened using the linear models for microarray data (LIMMA) method [18] in the Bioconductor R package (version 3.34.0; *http://www.bioconductor.org/packages/release/bioc/*

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Table 1. Differentially expressed genes and miRNAs between membranous nephropathy patients and controls.

Gene	logFC	*P*-value	miRNA	logFC	*P*-value
RFX7	1.92	6.28E-06	hsa-miR-126-3p	5.42	6.24E-05
ZNF800	1.41	1.18E-02	hsa-miR-30e-5p	4.12	6.53E-04
CCL5	1.34	1.86E-02	hsa-miR-145-5p	3.82	6.89E-04
BAX	1.30	3.64E-03	hsa-miR-143-3p	3.81	5.62E-03
ZNF532	1.29	3.05E-02	hsa-miR-214-3p	3.53	4.88E-03
MAPK11	1.29	4.81 E-04	hsa-miR-20a-5p	3.44	2.76E-02
CNOT6L	1.08	1.99E-04	hsa-miR-26b-5p	2.91	1.42E-02
BRSK1	1.04	1.60E-02	hsa-miR-195-5p	2.53	3.18E-02
MAPK15	1.03	2.55 E-03	hsa-miR-192-3p	1.55	2.54E-02
FBXO32	1.02	2.64E-03	hsa-miR-140-5p	1.47	9.10E-03
MBNL3	1.00	4.03E-02	hsa-miR-101-3p	1.23	1.54E-02
SEC24C	–1.88	8.48E-03	hsa-miR-328-5p	–3.15	4.54E-02
AKTIP	–1.42	1.86E-02	hsa-miR-125b-1-3p	–2.93	2.12E-03
PCMT1	–1.20	8.53E-03	hsa-miR-149-5p	–2.79	1.15E-02
RAB1A	–1.15	1.73E-04	hsa-miR-23a-5p	–2.76	3.93E-02
PPM1A	–1.14	3.49E-03	hsa-miR-324-3p	–2.44	2.84E-02
SLC13A3	–1.05	3.41E-02	hsa-miR-21-3p	–2.14	1.88E-02
CDK1	–1.36	4.79E-02	hsa-miR-320a	–1.37	1.84E-02

FC – fold change, miRNA – microRNA.

html/limma.html). The |log fold change (FC)| >1 and $P<0.05$ were set as the threshold values. A heatmap was created using R package pheatmap (version: 1.0.8; *http://cran.r-project.org/web/packages/pheatmap/index.html*) [19] to observe whether the use of DEGs and DEMs could distinguish the MN patients from the controls.

PPI network and module analyses

Protein-protein interaction (PPI) pairs were predicted by the Search Tool for the Retrieval of Interacting Genes (STRING, version 10.0; *http://www.string-db.org/*) [20]. Then, the PPI network of DEGs was constructed using Cytoscape software (version 3.4; *www.cytoscape.org/*) [21] based on the obtained PPI pairs. In the PPI network, the node score was calculated based on degree centrality. The node with the high score, which likely played an important role in the PPI network, was referred to as the hub protein. Following PPI network analysis, the significant modules were identified from the PPI network using the Molecular Complex Detection (MCODE; version 1.4.2, *http://apps.cytoscape.org/apps/mcode*) [22] plugin in the Cytoscape software [21] with degree cutoff ≥2 and k-core ≥3.

miRNA-target gene regulatory analysis

The target genes of DEMs were predicted using an online resource, miRDB (version 1.0; *http://mirdb.org*) [23]. Subsequently, the DEMs-target gene regulatory network was also constructed using Cytoscape software [21]. The DEGs identified in GSE73953 and the target genes of DEMs identified in GSE64306 were integrated to obtain the intersection genes between PBMCs and urine sediments samples.

Functional enrichment analyses

Gene Ontology (GO) biological process [24] and the Kyoto Encyclopedia of Genes Genomes (KEGG) [25] pathway enrichment analyses were carried out for all identified DEGs, the DEGs in modules, and the target genes of DEMs using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8; *http://david.abcc.ncifcrf.gov/*) [26] tool. A *P* value <0.05 was selected as the threshold value.

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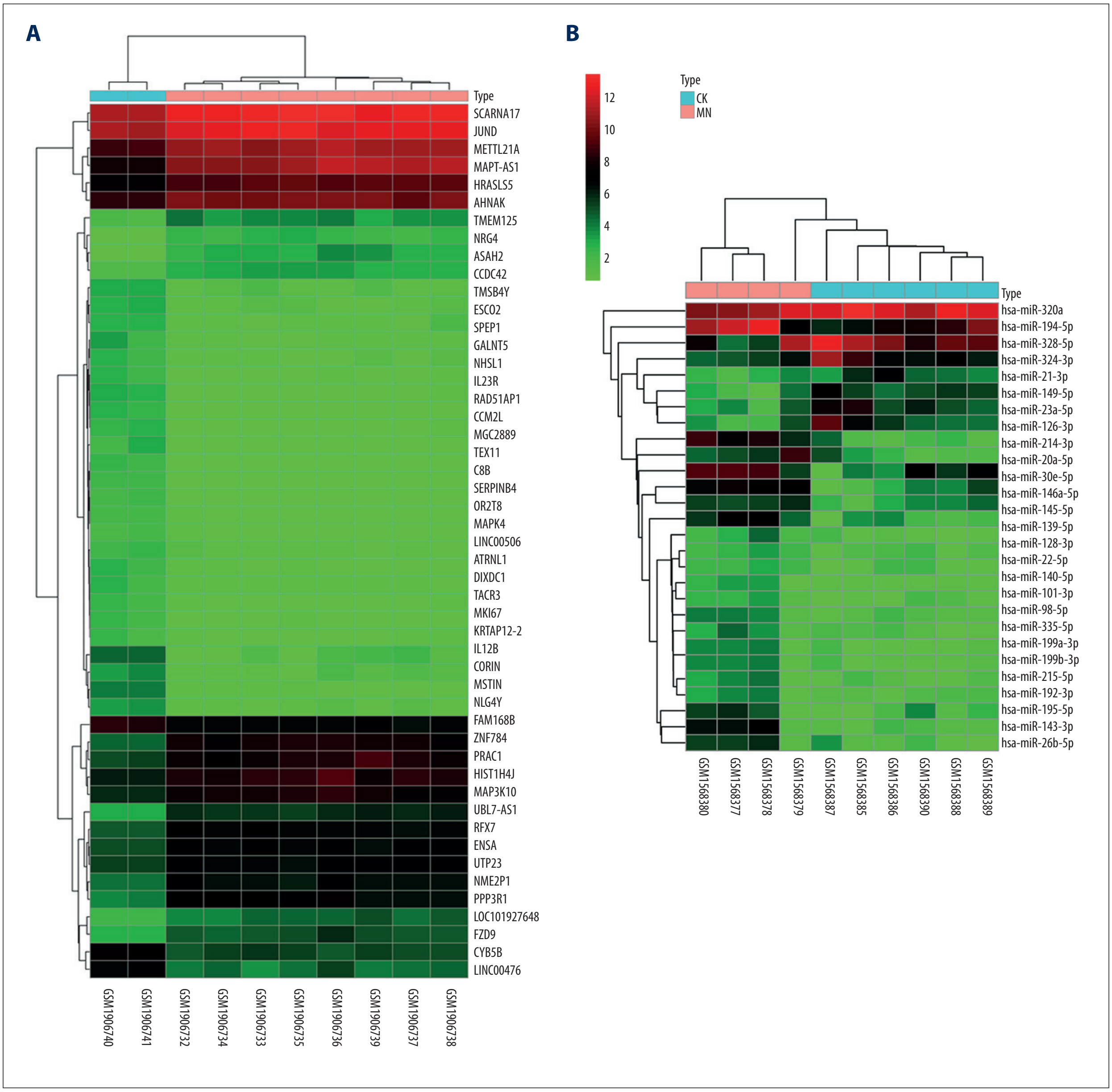

Figure 1. The hierarchical clustering heat maps of (**A**) differentially expressed genes (DEGs) and (**B**) differentially expressed miRNAs (DEMs) between membranous nephropathy (MN) patients and controls. MN patients and controls were completely separated by DEGs or DEMs.

Results

DEGs identification in PMBCs samples

After normalization, a total of 1246 DEGs were identified from PMBCs samples of MN patients compared with controls based on the thresholds of |logFC| >1 and *P*<0.05, including 643 upregulated (BRSK1, BR serine/threonine kinase 1; RFX7, regulatory factor X7; CNOT6L, CCR4-NOT transcription complex subunit 6 like; FBXO32, F-box protein 32; ZNF800, zinc finger protein 800; ZNF532, zinc finger protein 532; MBNL3, muscle blind like splicing regulator 3; CCL5, C-C motif chemokine ligand 5; MAPK11, mitogen-activated protein kinase 11; MAPK15) and 603 downregulated genes (PPM1A, protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1A; CDK1, cyclin dependent kinase 1; RAB1A, RAB1A, member RAS oncogene family; SEC24C, SEC24 homolog C, COPII coat complex component; PCMT1, protein-L-isoaspartate (D-aspartate) O-methyltransferase; AKTIP, AKT interacting protein; SLC13A3, solute carrier family 13 member 3) (Table 1). Heat map analysis showed that the MN and control groups were well distinguished by the DEGs (Figure 1A).

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Table 2. GO and KEGG pathway enrichment for differentially expressed genes.

	Term	*P*-value	Genes
Up	hsa05322: Systemic lupus erythematosus	4.54E-09	HIST1H2AB, HIST1H4L, HIST1H4K, HIST2H4B, C8G, C1QB, HIST2H2AB, HIST1H4B, HIST2H2AC, HIST1H4E, HIST1H2AH, HIST1H4F, HIST1H4C, HIST1H2AJ, HIST1H4D, HIST1H2AM, HIST1H4I, HIST1H2AL, HIST1H4J, HIST1H4H
	hsa05034: Alcoholism	4.36E-07	HIST1H2AB, HIST1H4L, HIST1H4K, CREB1, HIST2H4B, HIST2H2AB, HIST1H4B, HIST2H2AC, ATF6B, HIST1H4E, HIST1H2AH, HIST1H4F, HIST1H4C, HIST1H2AJ, HIST1H4D, HIST1H2AM, HIST1H4I, HIST1H2AL, HIST1H4J, HIST1H4H
	hsa04978: Mineral absorption	4.04E-06	MT1A, TRPM7, MT2A, MT1E, MT1B, SLC6A19, MT1H, MT1X, MT1G, MT1F
	hsa05203: Viral carcinogenesis	5.69E-05	HIST1H4L, HIST1H4K, CREB1, HIST2H4B, HIST1H4B, JUN, BAX, CASP8, ATF6B, HIST1H4E, HIST1H4F, IRF3, HIST1H4C, HIST1H4D, PRKACB, HIST1H4I, HIST1H4J, HIST1H4H
	hsa05020: Prion diseases	2.07E-03	C1QB, BAX, PRKACB, CCL5, PRNP, C8G
	hsa05033: Nicotine addiction	4.91E-03	GABRD, SLC32A1, GABRE, GABRA3, CHRNA4, GABRQ
	hsa04080: Neuroactive ligand-receptor interaction	2.36-02	GABRD, C3AR1, GABRE, CSH2, CYSLTR1, GABRA3, GH2, P2RY13, APLNR, LTB4R2, P2RY1, CHRNA4, ADRA2C, GABRQ, F2R
	hsa04950: Maturity onset diabetes of the young	3.53E-02	HES1, IAPP, PAX6, PAX4
	hsa03010: Ribosome	3.71E-02	RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS9, RPS21
	hsa05016: Huntington's disease	4.33E-02	ATP5D, NDUFB11, UQCR11, ATP5B, BAX, CREB1, CASP8, COX8A, DNAH2, REST, POLR2J2
	GO: 0045653~negative regulation of megakaryocyte differentiation	1.04E-11	HIST1H4L, HIST1H4K, HIST1H4B, HIST1H4E, HIST1H4F, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, HIST2H4B, HIST1H4H
	GO: 0016233~telomere capping	2.41E-10	HIST1H4L, HIST1H4K, HIST1H4B, HIST1H4E, HIST1H4F, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, HIST2H4B, HIST1H4H
	GO: 0071294~cellular response to zinc ion	7.58E-10	MT1L, MT1A, CREB1, MT2A, MT1E, MT1B, MT1H, MT1X, MT1G, MT1F
	GO: 0006336~DNA replication-independent nucleosome assembly	1.04E-09	HIST1H4L, HIST1H4K, HIST1H4B, HIST1H4E, HIST1H4F, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, HIST2H4B, HIST1H4H
	GO: 1904837~beta-catenin-TCF complex assembly	1.54E-09	KMT2D, HIST1H4L, HIST1H4K, HIST2H4B, HIST1H4B, HIST1H4E, HIST1H4F, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, TERT, HIST1H4H
	GO: 0006355~regulation of transcription, DNA-templated	2.49E-03	ZNF486, TMEM189-UBE2V1, ZNF532, PAX6, ZNF675, ZKSCAN1, PAX4, REST, SLIRP, ZNF253, ZBTB37, ZNF776, ZNF681, NKX6-3, ZNF492, ZNF720, ZNF576, ARGLU1, ZNF644, ZNF92, ZNF141, SIX5, MIXL1, ZNF2, ZNF37A, ZNF234, ASCL2, HES1, PRDM8, ZNF718, MSX1, ZNF784, ZNF785, KHSRP, FOXC1, ZNF713, ZNF740, KMT2D, ZNF800, TXLNG, ZNF780B, CHD9, RAX2, MEIS2, CNOT6L, CASZ1, ZNF124, TPRX1, ZNF625, VHL, CREB1, ZNF621, RFX7, ZNF626, WWTR1, ZNF524, TULP3, TCEB3C, RBAK, IRF3, ZNF117, ZNF257
	GO: 0043524~negative regulation of neuron apoptotic process	1.26E-02	FZD9, NRP1, SNCB, CLCF1, JUN, BAX, CRLF1, TERT, NDNF, F2R
	GO: 0007623~circadian rhythm	1.92E-02	JUN, CREB1, JUND, PAX4, ID4, AGRP, CPT1A
	GO: 0048844~artery morphogenesis	2.04E-02	HES1, NRP1, FOXC1, PRDM1
	GO: 0070493~thrombin receptor signaling pathway	2.51E-02	DGKQ, IQGAP2, F2R

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Table 2 continued. GO and KEGG pathway enrichment for differentially expressed genes.

	Term	*P*-value	Genes
Down	hsa04010: MAPK signaling pathway	8.72E-03	LAMTOR3, TAOK2, MAP2K3, PPM1A, ELK1, FGF23, NFKB1, HSPA1A, STK3, PRKACG, MAP3K4, HSPA2, RAP1A, CRK, CHUK, FGF3
	hsa05169: Epstein-Barr virus infection	1.07E-02	PRKACG, CDK1, GTF2E2, PSMC5, HSPA2, PSMC3, MAP2K3, PIK3CA, NFKB1, PIK3R5, HSPA1A, RBPJL, CHUK
	hsa05162: Measles	1.71E-02	HSPA2, EIF2S1, IFNA10, PIK3CA, NFKB1, PIK3R5, HSPA1A, IL12B, IFNGR2, CHUK
	hsa04920: Adipocytokine signaling pathway	1.72E-02	IRS2, ACSL1, SLC2A4, PRKAB1, NFKB1, ACSL6, CHUK
	hsa05145: Toxoplasmosis	2.37E-02	HSPA2, MAP2K3, PIK3CA, NFKB1, PIK3R5, HSPA1A, IL12B, IFNGR2, CHUK
	hsa05142: Chagas disease (American trypanosomiasis)	3.44E-02	PLCB3, PPP2CA, PIK3CA, NFKB1, PIK3R5, IL12B, IFNGR2, CHUK
	hsa04620: Toll-like receptor signaling pathway	3.76E-02	MAP2K3, IFNA10, PIK3CA, NFKB1, PIK3R5, IL12B, CHUK, SPP1
	hsa04015: Rap1 signaling pathway	4.68E-02	PLCB3, ADORA2A, MAP2K3, RAP1A, FGF23, PIK3CA, RAPGEF4, PIK3R5, LPAR1, KIT, CRK, FGF3
	hsa04115: p53 signaling pathway	4.80E-02	STEAP3, CDK1, RRM2, CCNG2, SESN1, SESN3
	hsa04062: Chemokine signaling pathway	4.99E-02	PRKACG, PLCB3, HCK, CXCR6, RAP1A, PIK3CA, NFKB1, PIK3R5, CXCR3, CRK, CHUK
	GO: 0033673~negative regulation of kinase activity	1.87E-03	AJUBA, IRS2, MSTN, PRDX3
	GO: 0033169~histone H3-K9 demethylation	3.52E-03	KDM1A, KDM7A, KDM3A, JMJD1C
	GO: 0046854~phosphatidylinositol phosphorylation	6.55E-03	INPP1, IRS2, PIP5KL1, PIP5K1B, FGF23, PIK3CA, PIK3R5, KIT, FGF3
	GO: 0042026~protein refolding	8.89E-03	HSPA2, HSPA1A, DNAJA4, DNAJA2
	GO: 0000186~activation of MAPKK activity	1.11E-02	LAMTOR3, MAP3K4, TAOK2, RAP1A, CRK, ADAM9
	GO: 0051091~positive regulation of sequence-specific DNA binding transcription factor activity	1.24E-02	KDM1A, PHB2, ARHGEF5, CYTL1, NEUROG1, KIT, PLPP3, STK3, ANXA3
	GO: 0030901~midbrain development	1.25E-02	KDM7A, RFX4, GDF7, WLS, FOXB1
	GO: 0009408~response to heat	1.32E-02	HSPA2, DNAJA1, MSTN, DNAJB4, DNAJA4, DNAJA2
	GO: 0055114~oxidation-reduction process	1.68E-02	STEAP3, GLRX5, SNCA, SESN1, SESN3, GLDC, KDM1A, ALDH1A3, FMO3, KDM3A, QSOX2, NSDHL, KDM7A, PTGR1, FA2H, CYB5RL, CYB5B, CYP27A1, DOHH, SDHC, RRM2, AOX1, HSD11B1, ASPHD1, DUS2, JMJD1C, CP, BCO2
	GO: 0071902~positive regulation of protein serine/threonine kinase activity	1.90E-02	ACSL1, PPP2CA, SNCA, LTF, STK3

GO – gene ontology; KEGG – Kyoto Encyclopedia of Genes Genomes. Top 10 were listed.

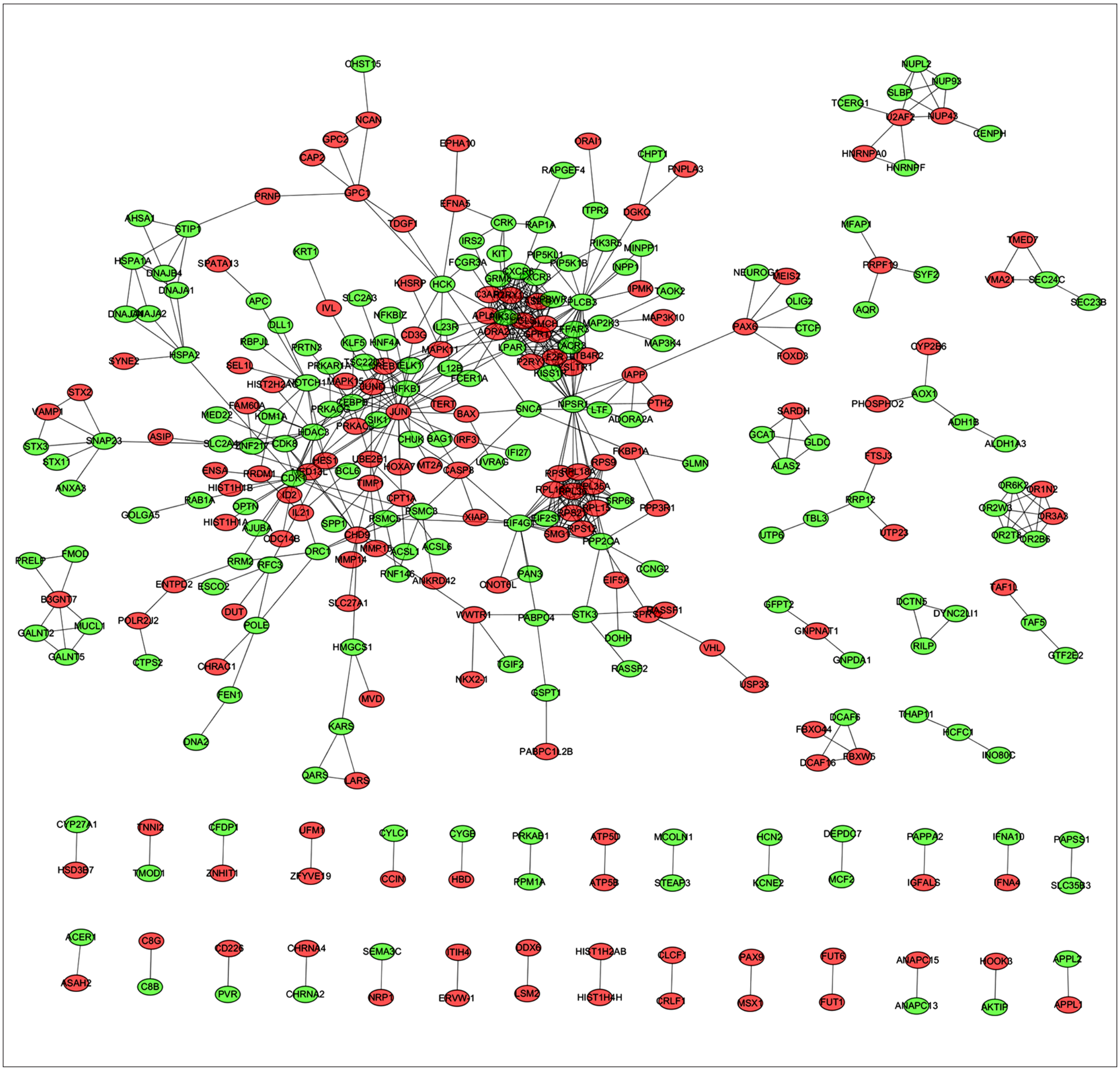

Figure 2. The protein-protein interaction network constructed by the differentially expressed genes. Red represents upregulated genes and green represents downregulated genes.

Functional enrichment analyses for DEGs in PMBCs samples

The upregulated DEGs were significantly enriched into 10 KEGG pathways (including Prion diseases, CCL5) and 43 GO biological process terms (including negative regulation of neuron apoptotic process, BAX), while the downregulated DEGs were significantly enriched into 12 KEGG pathways (including hsa04010: MAPK signaling pathway, PPM1A; hsa04115: p53 signaling pathway, CDK1), and 20 GO biological process terms (Table 2).

PPI network and module analyses for DEGs in PMBCs samples

A total of 621 PPI pairs (i.e., CDK1-RAB1A), were predicted from the DEGs, which were then used for constructing the PPI network, including 329 nodes (Figure 2). The hub proteins in the PPI network were screened by calculating the degree. The results (Figure 3) showed CDK1 (degree=18), CCL5 (degree=12), and MAPK11 (degree=8) may be crucial genes for MN.

From the PPI network, 3 significant modules were screened (Figure 4). Functional enrichment analyses revealed that the genes in module 2 were involved in hsa04062: chemokine

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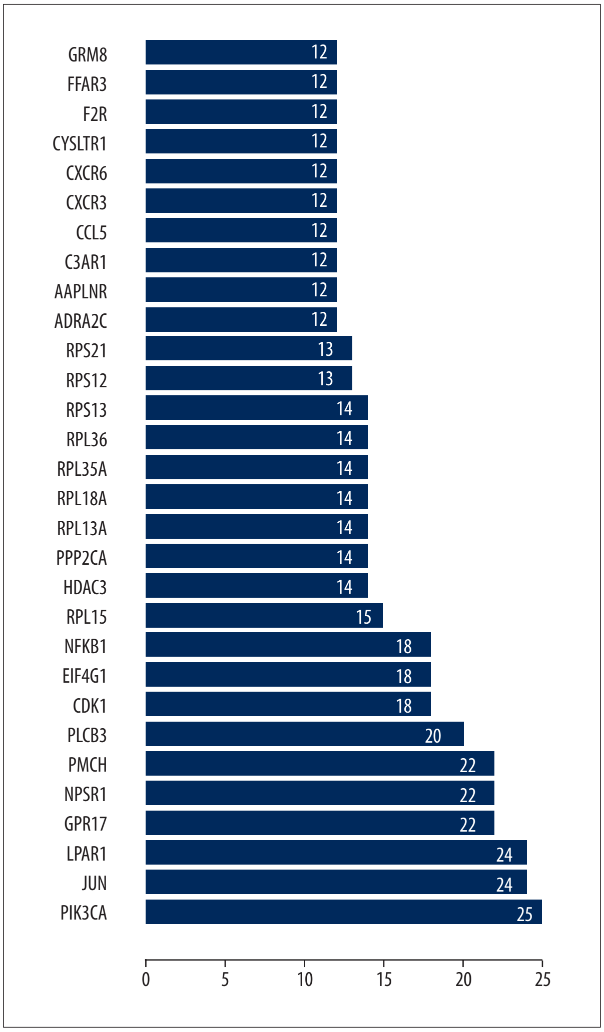

Figure 3. Ranking of nodes in protein-protein interaction (PPI) network to screen hub proteins.

signaling pathway (CCL5) and hsa04060: cytokine-cytokine receptor interaction (CCL5) (Table 3).

DEMs identification

A total of 28 DEMs, including 21 upregulated (including hsa-miR-214-3p, hsa-miR-192-3p, hsa-miR-20a-5p, and hsa-miR-195-5p) and 7 downregulated miRNAs (including hsa-miR-328-5p), were identified between urine sediments of MN patients and controls (Table 1). Heat map analysis showed that the MN group and the control group were also well distinguished by the DEMs (Figure 1B).

miRNA-target gene regulatory network analysis for DEMs

Using the miRDB database, 276 target genes of DEMs were predicted. The DEMs-target gene regulatory network was constructed as shown in Figure 5, with the miR-30e-5p, miR-195-5p, and miR-20a-5p regulating more target genes.

Functional enrichment analyses for DEMs

The functions of the target genes of DEMs were predicted by GO and KEGG enrichment analyses using DAVID. As shown in Table 4, these target genes were significantly enriched in 26 GO terms, such as cell cycle arrest (PPM1A), intracellular signal transduction (BRSK1), protein phosphorylation (BRSK1), and establishment of cell polarity (BRSK1).

Integrated analysis of 2 datasets

A total of 13 intersection genes were identified between DEGs in PMBCs and the target genes of DEMs in urine sediments, such as RFX7, MBNL3, SEC24C, FBXO32, ZNF800, ZNF532, RAB1A, AKTIP, SLC13A3, PPM1A, CNOT6L, PCMT1, and BRSK1 (Table 5). Among them, hsa-miR-214-3p-SEC24C, hsa-miR-192-3p (upregulated)-RAB1A (downregulated), hsa-miR-20a-5p (upregulated)-AKTIP (downregulated), hsa-miR-195-5p (upregulated)-SLC13A3 (downregulated), hsa-miR-195-5p (upregulated)-PPM1A (downregulated), and hsa-miR-328-5p (downregulated)-BRSK1 (upregulated) were negatively related in their expression level (Table 1).

Discussion

By integrating the mRNA expression profile data in PBMCs and miRNA expression profile data in urine sediments, our present study suggests that hsa-miR-195-5p, hsa-miR-192-3p, hsa-miR-328-5p, and their target genes PPM1A, RAB1A, and BRSK1 may be crucial biomarkers for the diagnosis and development of MN. PPM1A and BRSK1 may be involved in MN by participating in the MAPK related signaling pathway, while RAB1A may also be associated with MN by involving the p53 signaling pathway (CDK1-RAB1A).

Accumulating evidence has demonstrated that the MAPK pathway is frequently activated in nephropathy. Activated p38 MAPK mediates apoptosis of glomerular podocytes by increasing the Bax/Bcl-2 ratio [27,28]. Furthermore, activated extracellular signal-regulated kinase (ERK1/2) and p38 MAPK were also reported to induce an increase in the activity of nuclear factor-kappaB (NF-κB) and produce the expression of several pro-inflammatory cytokines, such as IL-6, IL-1, tumor necrosis factor (TNF)-alpha, and IL-17, resulting in renal injury, which could be reversed by their inhibitors [29,30]. Thus, inhibition of the MAPK signaling pathway has been suggested as an underlying treatment approach for nephropathy. This hypothesis has been investigated in several studies. For example, Zheng et al. demonstrated astragaloside IV restored podocyte morphology and cytoskeleton loss induced by complement membranous attack complex by reducing the phosphorylation of JNK and ERK1/2 and attenuated podocyte injury in MN [31].

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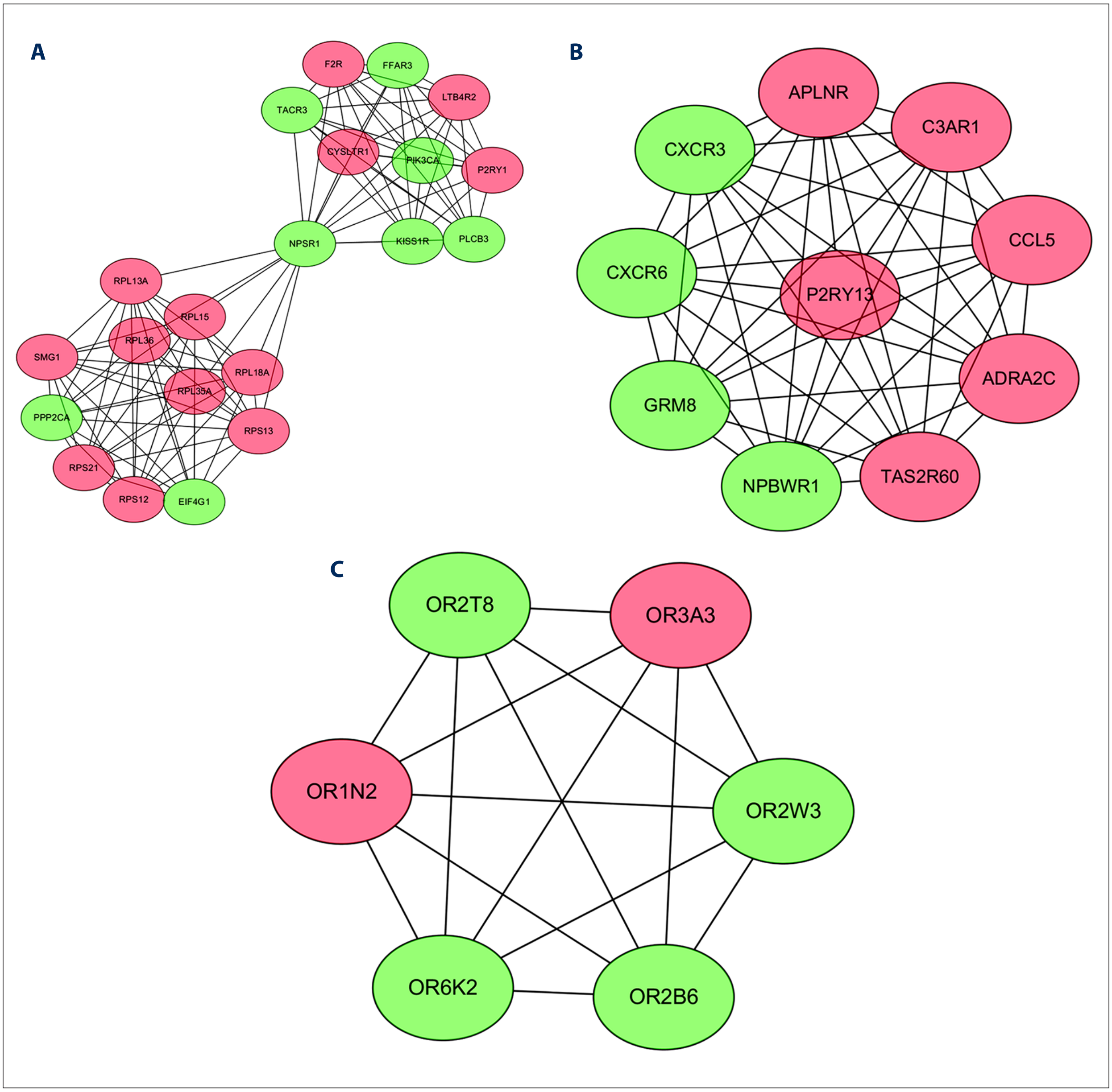

Figure 4. (**A–C**) Modules extracted from protein-protein interaction (PPI) network. Red represents upregulated genes and green represents downregulated genes.

Malik et al. observed the use of apigenin ameliorated streptozotocin-induced diabetic nephropathy in rats via preventing the activation of the MAPK pathway, which then inhibited inflammation (reduced TNF-α, IL-6, and NF-κB expression) and apoptosis (increased expression of Bcl-2 and decreased Bax and caspase-3) [32]. Similarly, pretreatment with galangin preserved renal function (normalized serum creatinine and blood urea nitrogen), suppressed oxidative stress (decreased level of malondialdehyde), inflammation (reduced levels of TNF-α and IL-6), and the activation of apoptotic pathways (decreased expression of Bax and caspase-3) by de-phosphorylating p38, JNK, and ERK1/2 proteins [33]. In our study, MAPK11, MAPK15 as well as pro-inflammatory cytokine CCL5 and pro-apoptotic Bax were also found to be significantly upregulated in MN, further verifying the MAPK-mediated inflammation and apoptosis mechanisms for MN. In addition, a recent study found that PPM1A could negatively regulate ERK by directly dephosphorylating its pT position early in epidermal growth factor stimulation [34]. Using a functional genomic approach, PPM1A was shown to dephosphorylate IKKβ at Ser177 and Ser181 and terminate IKKβ-induced NF-κB activation, leading to inflammation suppression [35]. Furthermore, genetic manipulation experiments demonstrated that knockdown of PPM1A expression accelerated the ability of monocytes to differentiate into

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Table 3. GO and KEGG pathway enrichment for genes in modules.

Module	Term	*P*-value	Genes
1	hsa03010: Ribosome	2.60E-08	RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	hsa04080: Neuroactive ligand-receptor interaction	5.56E-04	KISS1R, TACR3, CYSLTR1, LTB4R2, P2RY1, F2R
	hsa04020: Calcium signaling pathway	1.00E-03	PLCB3, TACR3, CYSLTR1, LTB4R2, F2R
	hsa04611: Platelet activation	4.32E-03	PLCB3, P2RY1, PIK3CA, F2R
	hsa04015: Rap1 signaling pathway	1.61E-02	PLCB3, P2RY1, PIK3CA, F2R
	hsa05142: Chagas disease (American trypanosomiasis)	2.93E-02	PLCB3, PPP2CA, PIK3CA
	hsa04071: Sphingolipid signaling pathway	3.82E-02	PLCB3, PPP2CA, PIK3CA
	GO: 0000184~nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	3.76E-17	EIF4G1, RPL35A, RPL18A, RPL13A, PPP2CA, RPL15, RPS12, RPS13, RPL36, SMG1, RPS21
	GO: 0006413~translational initiation	1.85E-12	EIF4G1, RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	GO: 0006614~SRP-dependent cotranslational protein targeting to membrane	1.00E-11	RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	GO: 0019083~viral transcription	3.51E-11	RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	GO: 0006412~translation	2.56E-10	EIF4G1, RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	GO: 0006364~rRNA processing	3.33E-09	RPL35A, RPL18A, RPL13A, RPL15, RPS12, RPS13, RPL36, RPS21
	GO: 0007200~phospholipase C-activating G-protein coupled receptor signaling pathway	1.01E-06	KISS1R, CYSLTR1, LTB4R2, P2RY1, F2R
	GO: 0007218~neuropeptide signaling pathway	2.24E-04	KISS1R, CYSLTR1, LTB4R2, NPSR1
	GO: 0002181~cytoplasmic translation	3.98E-04	RPL35A, RPL15, RPL36
	GO: 0061737~leukotriene signaling pathway	7.13E-03	CYSLTR1, LTB4R2
	GO: 0030168~platelet activation	8.15E-03	P2RY1, PIK3CA, F2R
	GO: 0007204~positive regulation of cytosolic calcium ion concentration	1.09E-02	CYSLTR1, P2RY1, F2R
	GO: 0051281~positive regulation of release of sequestered calcium ion into cytosol	3.17E-02	NPSR1, F2R
	GO: 0098609~cell-cell adhesion	4.07E-02	EIF4G1, PLCB3, RPL15
2	hsa04080: Neuroactive ligand-receptor interaction	1.10E-05	P2RY13, C3AR1, APLNR, GRM8, NPBWR1, ADRA2C
	hsa04062: Chemokine signaling pathway	2.29E-02	CXCR6, CXCR3, CCL5
	hsa04060: Cytokine-cytokine receptor interaction	3.40E-02	CXCR6, CXCR3, CCL5
	GO: 0007186~G-protein coupled receptor signaling pathway	4.04E-08	TAS2R60, C3AR1, APLNR, NPBWR1, CXCR6, ADRA2C, CXCR3, CCL5
	GO: 0006935~chemotaxis	3.04E-05	C3AR1, CXCR6, CXCR3, CCL5
	GO: 0070098~chemokine-mediated signaling pathway	6.23E-04	CXCR6, CXCR3, CCL5

Table 3 continued. GO and KEGG pathway enrichment for genes in modules.

Module	Term	*P*-value	Genes
	GO: 0006954~inflammatory response	8.66E-04	C3AR1, CXCR6, CXCR3, CCL5
	GO: 0010759~positive regulation of macrophage chemotaxis	5.88E-03	C3AR1, CCL5
	GO: 0071407~cellular response to organic cyclic compound	3.12E-02	P2RY13, CCL5
	GO: 0060326~cell chemotaxis	3.43E-02	C3AR1, CCL5
	GO: 0050796~regulation of insulin secretion	3.54E-02	ADRA2C, CCL5
3	hsa04740: Olfactory transduction	6.27E-07	OR2B6, OR3A3, OR6K2, OR1N2, OR2T8, OR2W3
	GO: 0050911~detection of chemical stimulus involved in sensory perception of smell	1.01E-08	OR2B6, OR3A3, OR6K2, OR1N2, OR2T8, OR2W3
	GO: 0007186~G-protein coupled receptor signaling pathway	4.35E-07	OR2B6, OR3A3, OR6K2, OR1N2, OR2T8, OR2W3
	GO: 0007608~sensory perception of smell	8.74E-04	OR2B6, OR2T8, OR2W3
	GO: 0050907~detection of chemical stimulus involved in sensory perception	2.80E-02	OR6K2, OR1N2

GO – gene ontology; KEGG – Kyoto Encyclopedia of Genes Genomes.

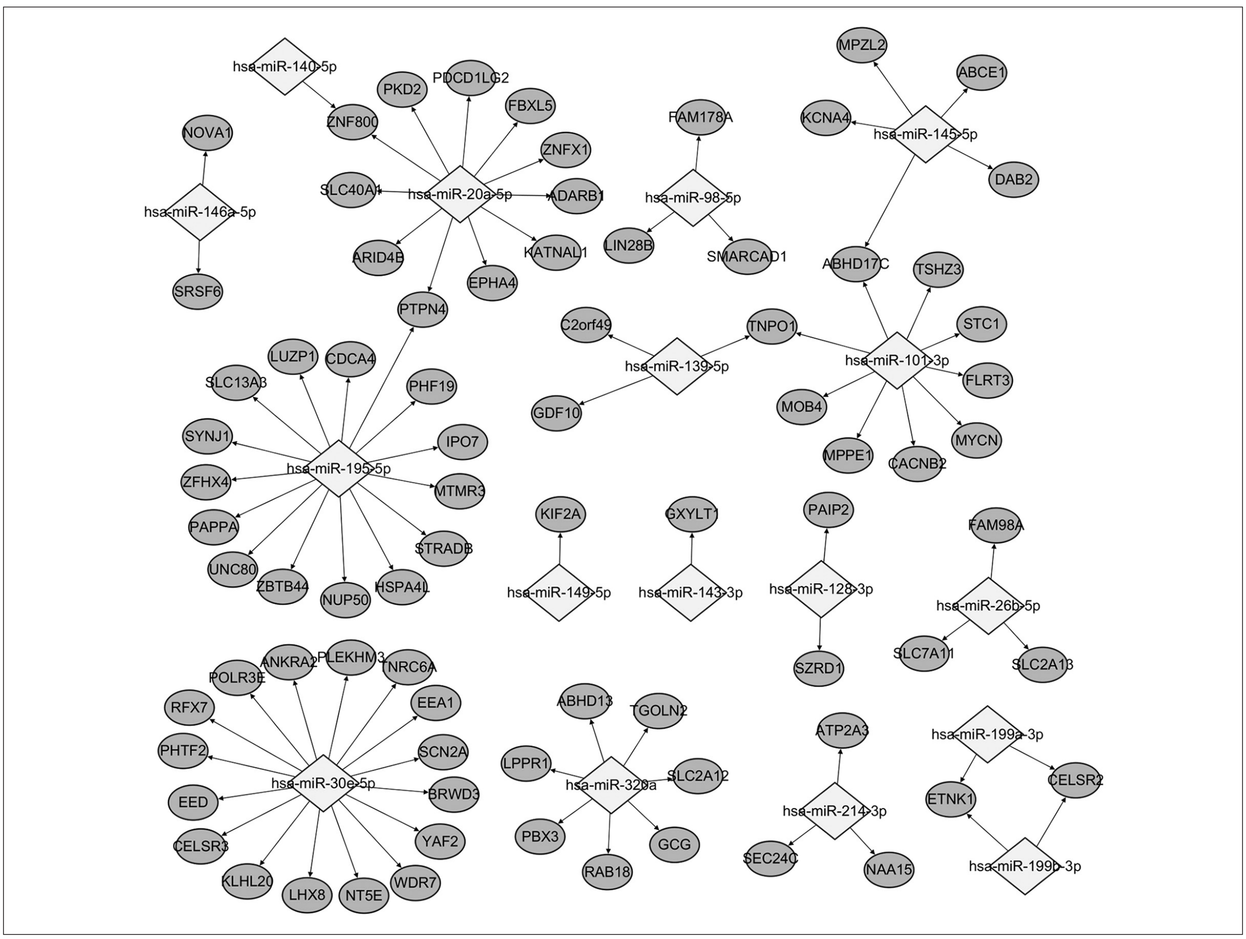

Figure 5. The microRNA (miRNA)-target gene regulatory networks. Ellipse represents genes and rhombus represents miRNAs.

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Table 4. GO and KEGG pathways enrichment for target genes of differentially expressed miRNAs.

Term	*P*-value	Genes
hsa04550: Signaling pathways regulating pluripotency of stem cells 6	2.99E-02	SMARCAD1, PCGF5, ACVR2A, FZD4, AKT3, FZD6
GO: 0045893~positive regulation of transcription, DNA-templated	4.09E-04	SMARCAD1, TBL1XR1, BTRC, TGFBR1, NAA15, PPM1A, RORA, FZD4, MYCN, CCNE1, RNF6, NPAS2, DAB2, LBH, MAP3K2, YAF2, SALL1, RFX3, INSR
GO: 0035556~intracellular signal transduction	1.90E-03	TGFBR1, STK17B, SPSB4, AKAP13, BRSK1, AKAP11, MARK1, RGL2, STAC, TLK1, STK39, MASTL, STK38L, AKT3, IGFBP5
GO: 0006468~protein phosphorylation	5.67E-03	TGFBR1, STK17B, AKAP13, STRADB, PXK, BRSK1, MARK1, CCNE1, MAPK6, MAP3K2, MAP3K1, STK39, TLK1, STK38L, AKT3
GO: 0007179~transforming growth factor beta receptor signaling pathway	9.94E-03	USP9X, ZFYVE9, TGFBR1, KLF10, GDF10, MTMR4
GO: 0006511~ubiquitin-dependent protein catabolic process	1.50E-02	RNF6, USP3, USP9X, BTRC, UBE4B, FBXO32, AMFR, USP25
GO: 0007050~cell cycle arrest	1.53E-02	TGFBR1, PKD2, PPM1A, STRADB, UHMK1, VASH1, RRAGB
GO: 0000226~microtubule cytoskeleton organization	1.82E-02	DYNC1LI2, CLASP2, MARK1, WEE1, EML4
GO: 0006349~regulation of gene expression by genetic imprinting	2.11E-02	ARID4A, ARID4B, EED
GO: 0023014~signal transduction by protein phosphorylation	2.43E-02	ACVR2A, TGFBR1, STK39, INSR
GO: 0045892~negative regulation of transcription, DNA-templated	2.59E-02	TSHZ3, CIPC, DAB2, ARID4A, YAF2, BTRC, KLF10, SALL1, NAB1, EED, RUNX1T1, RFX3, ZEB1, PPARGC1B
GO: 0035904~aorta development	2.64E-02	NDST1, PKD2, LRP2
GO: 0006351~transcription, DNA-templated	2.69E-02	TSHZ3, ZNF532, EZH1, ARID4B, ZNF800, NAA15, CBX2, ZEB1, RORA, ZNF654, PCGF5, CIPC, NPAS2, EPC2, LBH, CDYL, CNOT6L, ZNF326, PHTF2, EED, CC2D1B, LHX8, TBL1XR1, KLF10, RUNX1T1, ZBTB44, POLR3E, MYCN, MED19, ZFHX4, PHF19, BCORL1, YAF2, RNF2, SALL1, DR1, NAB1, RFX3, LCOR
GO: 0016575~histone deacetylation	2.73E-02	TBL1XR1, ARID4A, SALL1, ARID4B
GO: 0000122~negative regulation of transcription from RNA polymerase II promoter	2.77E-02	TBL1XR1, TSHZ3, USP3, CPEB3, KLF10, USP9X, PPM1A, CBX2, ZEB1, PHF19, SALL1, RNF2, DR1, VEGFA, EED, CC2D1B, NFX1, VLDLR
GO: 0008045~motor neuron axon guidance	2.92E-02	ALCAM, EPHA4, KIF5C
GO: 0010862~positive regulation of pathway-restricted SMAD protein phosphorylation	3.05E-02	ACVR2A, DAB2, TGFBR1, GDF10
GO: 0071560~cellular response to transforming growth factor beta stimulus	3.21E-02	ZFP36L2, TGFBR1, FBN1, PPM1A
GO: 0018105~peptidyl-serine phosphorylation	3.27E-02	TGFBR1, STK39, MASTL, STK38L, UHMK1, AKT3
GO: 0006470~protein dephosphorylation	3.37E-02	MTMR3, BTRC, PTPN4, PPM1A, PPM1B, MTMR4
GO: 0060078~regulation of postsynaptic membrane potential	3.84E-02	SCN2A, PKD2, SCN9A
GO: 0048675~axon extension	4.50E-02	SLC9A6, USP9X, ULK2
GO: 0030010~establishment of cell polarity	4.50E-02	BRSK1, MARK1, WEE1
GO: 0007507~heart development	4.58E-02	FLRT3, ADM, TGFBR1, SALL1, FBN1, PKD2, AKAP13
GO: 0030335~positive regulation of cell migration	4.68E-02	DAB2, F3, TGFBR1, PTP4A1, VEGFA, INSR, ATP8A1
GO: 0006661~phosphatidylinositol biosynthetic process	4.91E-02	MTMR3, SYNJ1, PIP4K2A, MTMR4
GO: 0016055~Wnt signaling pathway	4.99E-02	CCNE1, DAB2, BTRC, PPM1A, CELSR2, FZD4, MARK1

GO – gene ontology; KEGG – Kyoto Encyclopedia of Genes Genomes; miRNAs – microRNAs.

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Table 5. The intersection genes between peripheral blood mononuclear cells and urine sediments and their regulatory miRNAs.

miRNA name	Gene symbol
hsa-miR-30e-5p	RFX7
hsa-miR-30e-5p	MBNL3
hsa-miR-214-3p	SEC24C
hsa-miR-214-3p	FBXO32
hsa-miR-140-5p	ZNF800
hsa-miR-101-3p	ZNF532
hsa-miR-192-3p	RAB1A
hsa-miR-20a-5p	ZNF800
hsa-miR-20a-5p	AKTIP
hsa-miR-195-5p	SLC13A3
hsa-miR-195-5p	PPM1A
hsa-miR-195-5p	CNOT6L
hsa-miR-195-5p	PCMT1
hsa-miR-328-5p	BRSK1

inflammatory (M1) macrophages and promoted the production of inflammatory cytokines [36]. Thus, PPM1A may be hypothesized to be downregulated and promote the development of MN by MAPK-mediated inflammation and apoptosis, which was preliminarily proved in our study. However, there was no study to confirm the roles of PPM1A in MN, and thus, further experiments are required. Furthermore, the study of Wang et al. revealed that the expression of BRSK1 was decreased in breast cancer cell lines, and treatment of PI3K inhibitor LY294002 could increase BRSK1 expression, implying BRSK1 may function as a cell apoptosis promotor via PI3K-Akt signaling pathway. Generally, there is crosstalk between the MAPK pathway and the PI3K pathways that plays a role in diseases [37]. Therefore, BRSK1 may also be involved in the MAPK pathway as well as other genes (i.e., MAPK6, MAP3K2, and MAP3K1) enriched in protein phosphorylation. As expected, our study showed BRSK1 was upregulated in MN patients and may cause the apoptosis of glomerular podocytes.

Although the RAB1A gene was not enriched into any pathway or GO term in our study, it could interact with CDK1 as seen from the PPI analysis and hereby we speculate that RAB1A may play important roles in MN by participating in the p53 signaling pathway which was CDK1 enriched. CDK1 encodes a cyclin kinase that associates with cyclin to control cell cycle transition from G1 to S and promote cell proliferation [38]. Thus, CDK1 may be downregulated in nephropathy [39]; this was also demonstrated by our study in the MN group. Due to interaction with CDK1, RAB1A may also be downregulated in MN as our study reported. This suggested finding can be indirectly demonstrated by other recent studies: Liu et al. found RAB1A was significantly lower in aging renal glomerular mesangial cells [40].

In addition to genes, miRNAs have also been suggested as key biomarkers in MN [13,15,41]. Thus, we also investigated the DEMs in urine sediments of MN patients. In line with the expression in the peripheral blood samples of MN patients [41] and amniotic fluid exosomes from fetuses with congenital hydronephrosis [42], our results also showed miR-195-5p was high expressed in urine sediments of MN patients. Transfection with miR-195 has been reported to reduce the protein levels of BCL2 and contribute to podocyte apoptosis via an increase in caspase-3 [43], while the use of miRNA-195 inhibitor protected mesangial cells from apoptosis and promoted cellular proliferation *in vitro* [44]. In accordance with these findings, our results also suggested miR-195 could negatively regulate PPM1A which was pro-inflammatory and pro-apoptotic when downregulated as we described. However, the relationship between miR-195 and PPM1A has never been validated in *in vitro* or *in vivo* experiments, and thus needs further investigation. Previously, miR-192 has been reported to be a potential biomarker of ischemia-reperfusion-induced kidney injury because of its increased expression in plasma and urine [45,46]. Also, its high expression has been demonstrated to be associated with the activation by p53 [47], while p53 upregulation suppressed the expression of CDK1 [48] and may also block RAB1A. In accordance with these aforementioned studies, we also found hsa-miR-192-3p was upregulated and could negatively regulate the expression of its target gene RAB1A. However, the relationship between miR-192-3p and RAB1A has never been validated in *in vitro* or *in vivo* experiments, and thus needs further investigation. MiR-328-5p was observed to be downregulated in pressure-induced renal fibrosis and it may function by upregulating its target gene CD44 expression [49]. Renal fibrosis is a pathological characteristic of advanced MN and therefore miR-328-5p may also be downregulated in MN, which was shown to be the case in our study. There have been no studies that have indicated a negative relationship between miR-328 and BRSK1 in MN, which was thus a novel result in our study.

Conclusions

Our study revealed that miR-195-5p, miR-192-3p, miR-328-5p, and their target genes PPM1A, RAB1A, and BRSK1 in peripheral blood and urine samples may be potential biomarkers for MN. Further clinical and experimental studies are needed to verify the diagnosis accuracy and interaction mechanism.

Conflict of interests

None.

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