Profiling and initial validation of urinary microRNAs as biomarkers in IgA nephropathy

Nannan Wang, Ru Bu, Zhiyu Duan, Xueguang Zhang, Pu Chen, Zuoxiang Li, Jie Wu, Guangyan Cai, Xiangmei Chen

Background: MicroRNAs (miRNAs) have been found in virtually all body fluids and used successfully as biomarkers for various diseases. Evidence indicates that microRNAs have important roles in IgA nephropathy (IgAN), a major cause of renal failure. However, the global urinary profile of miRNAs in patients with IgAN is unkown.
Methods: Microarray and RT-qPCR were sequentially used to screen and further verify miRNA expression profiles in urine sediments of IgAN patients in two independent cohorts. The control groups included both healthy subjects and non-IgAN patients. The targets of candidate miRNA were predicted using miRWalk algorithm, followed by function and pathway analysis using the databases of Gene Ontology and the KEGG pathway.
Results: At the screening stage, SAM analysis showed that miRNAs were in different levels only in specific IgAN subgroups. In IgAN I-II, a total of 128 miRNAs were significantly differentially expressed (fold change>2 and $P<0.05$). Hierarchical clustering showed a general distinction between IgAN I-II and control samples in a heat map. Four candidate miRNAs (miR-223-3p, miR-629-5p, miR-3613-3p and miR-4668-5p) were verified by RT-qPCR. At the validation stage, RT-qPCR results showed that the level of miR-223-3p was significantly higher, while miR-3613-3p was significantly lower in IgAN I-II than in control groups (all $P<0.05$). Bioinformatics analysis revealed that miR-223 was mainly involved in positive regulation of locomotion, transmembrane transport, chemical homeostasis, and cellular response to hormone stimulus.
Conclusions: The expression profile of miRNAs was significantly altered in IgAN, and the differentially expressed miRNAs, especially miR-223-3p and miR-3613-3p, might be potential non-invasive biomarkers of IgAN at an early stage. The miRNA-target database may provide a novel understanding of the pathogenesis of IgAN.

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Introduction

Immunoglobulin A nephropathy (IgAN), the most common type of primary glomerulonephritis worldwide, is characterized by a predominant deposition of IgA-containing immune complexes in the glomerular mesangium (Wyatt & Julian 2013). The diagnosis of IgAN relies entirely on a renal biopsy, which is invasive and cannot be frequently repeated in the same patient. The prognosis of this disease is good in the early stages, but over the next 20 years, up to 40% of affected patients will develop irreversible end-stage renal disease (ESRD) (Schena 1990). Therefore, the development of non-invasive biomarkers at an early stage would be of great significance for preventing or controlling its progression and reducing the probability of ESRD.

In recent years, the role of microRNAs (miRNAs) in a variety of physiological and pathophysiological processes has received much attention. MiRNAs are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level (Esteller 2011). It has been demonstrated that miRNAs exhibit functional dysregulation in IgAN (Chandrasekaran et al. 2012; Szeto & Li 2014). As miRNAs are easily accessible, relatively stable and resistant to RNAse-mediated degradation in body fluids (Chen et al. 2008), they have the potential to be used as non-invasive biomarkers.

Recent studies have shown that urinary levels of several selected miRNAs were significantly changed in patients with IgAN compared with healthy individuals (Wang et al. 2010b; Wang et al. 2011). However, to date, no data are available concerning the global urine profile of miRNAs in IgAN patients, and a study with both healthy controls and disease controls is lacking. Therefore, the aim of this study is to analyze the expression pattern of miRNAs in urine sediments associated with IgAN and to explore their possible roles in IgAN through bioinformatics analysis.

Materials and Methods

Patients and urine sample preparation

Patients who received renal biopsy from cember 2013 to November 2014 in the Nephrology Department at the Chinese PLA General Hospital were recruited for study. Inclusion criteria were: (1) primary glomerulonephritis; (2) age 20–50; (3) first renal biopsy, with eight or more glomeruli in biopsy tissues. Patients with other coexisting diseases, such as chronic hepatic diseases and diabetes, were excluded. All IgAN patients were pathologically classified to grades I–V by light microscopy according to the grading system of Lee et al (Lee et al. 2). Clinical data, including serum creatinine) and 24-hour urine protein excretion (UPE) were recorded at the time of kidney biopsy.

A whole-stream early morning urine specimen was collected at the day of renal biopsy. The urine sample was centrifuged at 3,000 g for 30 min and at 13,00 for 5 min at 4°C. Supernatant was discarded. Then the urinary cell pellet was lysed by RNA lysis buffer (TIANGEN, a) and stored at –80°C il used.

The study was carried out in accordance with the Declaration of Helsinki for Human Research and approved by the Ethics Committee of the Chinese PLA eral Hospital. Written informed consent for inclusion was obtained from all of the participants.

RNA extraction, miRNA microarray and RT-qPCR

Total RNA was extracted using miRcute miRNA Isolation Kit (TIANGEN,) according to the manufacturer's protocol. The quantity (ng/ml) and purity (A260/280 ratio) of the RNAs obtained was evaluated by NanoDrop 2000 spectrophotometer (Thermo-Scientific).

Affymetrix GeneChip miRNA 4.0 Arrays, which cover all of the 2,578 mature human miRNAs available in miRBase version 20 (June 24, 2013, www.mirbase.org/)(Kozomara & Griffiths-Jones 2011), was used to profile miRNA expressions. Briefly, 1 μg of RNA was polyA tailed and labelled with a FlashTag Biotin HSR Labeling Kit. The labelled RNA was hybridized at 48°C for 16 hr on the miRNA arrays, which were washed and stained with Affymetrix Fluidics Station 450 and scanned with an Affymetrix GeneChip Scanner 3000 using the Command Console software (Affymetrix, Santa Clara, CA). The data were analyzed with miRNA QCTool using the Affymetrix default analysis settings and quantile as the normalization method.

Real-time quantitative polymerase chain reaction (RT-PCR) was carried out to verify the candidate miRNAs revealed by microarray. Briefly, reverse transcription was performed using miRNA specific primers and a miRcute miRNA First-Strand cDNA Synthesis Kit (TIANGEN, China) according to the manufacturer's protocol. RT-qPCR was performed in duplicate on an ABI 7500HT (Applied Biosystems, CA, USA) using a miRcute miRNA qPCR Detection Kit (TIANGEN, China), according to the manufacturer's instructions. All of the primers were purchased from TIANGEN Biotech Company (Beijing, China). All PCR reactions were performed in triplicate, followed by melt curve analysis to verify their specificity and identity. U6 was selected as the endogenous reference control (Mestdagh et al. 2009). Relative miRNA expression levels were calculated using the -ΔΔCt method as previously described(Livak & Schmittgen 2001).

miRNA target prediction and function analysis

The differentially expressed miRNAs were imported into the miRWalk algorithm(Dweep et al. 2011) (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), and the prediction of their target genes was performed using nine additional algorithms, including TargetScan, DIANAmT, miRanda, miRDB, RNAhybrid, PICTAR4, PICTAR5, PITA and RNA22. Only miRNA targets identified by at least six algorithms were included in further analysis.

To explore the functional annotation and pathway enrichment of those predicted genes, the Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) database analyses were conducted using a DAVID online analysis tool(Huang da et al. 2009) (http://david.abcc.ncifcrf.gov/tools.jsp), in which we focused on the GO Biological Processes (BP) feature.

Statistical analysis

The microarray data were analyzed by the algorithm of SAM (www-stat.stanford.edu/~tibs/SAM/)(Olson 2006). The false discovery rate (FDR) was set to <0.05 and the minimum fold change (FC) was set to 2.0. Hierarchical clustering was carried out using the MeV 4.9 software (Multi Experiment Viewer, http://www.tm4.org/mev.html) to generate both miRNA and sample trees based on Pearson correlation. For

the RT-qPCR data, the Mann-Whitney test for two unpaired groups was executed by SPSS v20.0. $P<0.05$ was considered to be statistically significant. In bioinformatics analysis, the Fisher's exact test and χ^2 test were used to select the significant GO category or KEGG pathway, and the FDR was calculated to correct the *P* value. A *P*-value <0.05 was considered to be statistically significant.

Results

Patients Characteristics

The classes of the IgAN pathological grades were predominantly II, III, and IV, while grades I and V were rare. Accordingly, we grouped IgAN I–II as the early pathological change group; IgAN III as the mild pathological change group; and IgAN IV–V as the severe group.

Eighteen IgAN patients were selected for a screening cohort: 6 IgAN I–II, 6 IgAN III and 6 IgAN IV–V. Six healthy volunteers with negative urinalysis results were used as healthy controls. In addition, 4 patients with minimal change disease (MCD) and 4 with membranous nephropathy (MN) were used as disease controls.

A validation cohort with 102 additional IgAN patients, including 29 IgAN I–II, 52 IgAN III and 21 IgAN IV–V, was used to analyze the expression of candidate miRNAs. For this second miRNA analysis, we included 34 healthy volunteers, 27 MCD patients and 41 MN patients. Demographic and clinical characteristics of both the screening and validation cohorts at the time of renal biopsy are provided in Table 1.

Differentially expressed miRNAs in the screening cohort

The microarray data have been deposited in Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE64306. We compared the different subgroups of IgAN (I-II, III or IV-V) with both healthy and disease controls. The differentially expressed miRNAs were shown in the form of Volcano Plots (Figure 1) and Venn Diagrams (Figure 2). Interestingly, there were no

overlaps in changes to miRNAs for IgAN IV-V group as shown in the Venn diagram (Figure 2C). IgAN I-II and III groups shared dysregulation of only two miRNAs (miR-3613-3p and miR-4668-5p). It turned out that miRNAs were in different levels only in specific IgAN subgroups.

Since Grades I-II stands for an early stage of IgAN, we focused our study on the differentially expressed miRNAs in the IgAN I-II group. Firstly, the Venn diagram (Figure 2A) shows that a total of 128 miRNAs showed different levels in IgAN I-II patients compared with healthy subjects or non-IgAN patients. Supervised hierarchical clustering demonstrated that the 128 differentially expressed miRNAs achieved good separation between IgAN I-II samples and the controls (Figure 3). Secondly, the Venn diagram (Figure 2A) also shows an overlap of four miRNAs which differentiates IgAN I-II and all of the control groups. Two of them were in high levels (miR-223-3p and miR-629-5p), while two of them were in low levels (miR-3613-3p and miR-4668-5p). The 4 miRNAs were initially verified by RT-qPCR re-examination and revealed similar expression patterns with the microarray results (Figure S1).

Analysis of the candidate miRNAs in the validation cohort

For confirmation purposes, the 4 above-mentioned miRNAs (miR-223-3p, miR-629-5p, miR-3613-3p and miR-4668-5p) were selected as candidates and analyzed in the validation cohort. Thereafter, two candidates (miR-629-5p and miR-4668-5p) that showed less significant expression changes were further excluded (Figure S2). The other two miRNAs (miR-223-3p and miR-3613-3p) had the same pattern of expression as in the microarray results (Figure 4).

The level of miR-223-3p was significantly higher in patients with IgAN I-II than in normal controls (P = 0.004) and in patients with non-IgAN (all P < 0.01) (Figure 4A), while the level of miR-3613-3p was significantly lower in IgAN I-II in normal controls (P < 0.001) and in patients with non-IgAN (all P < 0.05) (Figure 4C).

When considering differences among IgAN subgroups, miR-223-3p was found to be higher in IgAN I-II vs III [2.29(1.16-4.63) vs 1.41(0.34-6.3), P = 0.037] (Figure 4B), and miR-3613-3p was found to be lower in

IgAN III vs IV-V [0.24(0.14-2.02) vs 0.31(0.13-3.61), P = 0.047] (Figure 4D).

Bioinformatics analysis of candidate miRNAs

According to the results of miRWalk, there were 343 predicted target genes of miR-223. However, only 3 out of 10 algorithms of miRWalk (DIANAmT, miRDB and Targetscan) can query the predicted target genes of miR-3613. Then we focused our study on miR-223. After enrichment analysis of the GO Biological Process and KEGG pathway, the top twenty GO Biological Process and KEGG pathways were annotated, as shown in Figure 5.

According to the GO analysis, the putative target genes of differentially expressed miRNAs in IgAN I-II were mainly related to such as sodium ion transport, positive regulation of locomotion and cell migration, response to insulin stimulus. According to KEGG pathway enrichment analysis, the putative targets were found to participate in Prostate cancer and Adipocytokine signaling pathway.

Discussion

To date, only two publications have profiled miRNAs expression in IgAN patients. One study focused on the peripheral blood mononuclear cells (PBMCs) and identified 37 miRNAs differentially expressed in IgAN patients compared with healthy persons (Serino et al. 2012). The other study focused on the kidney biopsy tissues and observed a total of 85 miRNAs that were differentially expressed in IgAN patients compared with normal tissues (Tan et al. 2013). However, the differentially expressed miRNAs identified in the two studies were not completely consistent with each other, which may be due to the use of different tissues and/or the possible effect of the different races of the subjects. In another study investigating selected urinary miRNAs, Wang et al.(Wang et al. 2011) found that the levels of miR-146a and miR-155 of IgAN were significantly higher than those of healthy controls. Nevertheless, none of the studies explored the whole urinary miRNA profile in IgAN patients. Moreover, neither did these studies enroll non-IgAN patients as disease controls, nor did they pay attention to the subtle discriminations among different grades of severity of IgAN.

In the present study, we investigated the expression levels of miRNA profile in urine sediments from IgA and non-IgA patients and healthy volunteers. The pathological grades of IgAN patients enrolled in this study were predominantly II, III, and IV according to Lee's grading system, while grades I and V were rare. The reasons for this result may be as follows: Firstly, Grade I corresponded to the earliest stage of IgAN and was hidden in the clinic due to untimely treatment or insufficient medical attention. Secondly, Grade V was the most severe stage of IgAN in which the renal cortex of bilateral kidneys were always too thin to undergo renal puncture biopsies. Therefore, these patients rarely underwent biopsy due to increased risk of bleeding complications.

By the two step screening and validation approach, we profiled the expression patterns of miRNAs and identified two miRNAs (miR-223-3p and miR-3613-3p) which were significantly different in early stage IgAN. These results indicates that the levels of miR-223-3p and miR-3613-3p may have an important potential for diagnostic value at an early stage of IgAN. Recent evidences have shown that certain miRNAs, such as miR-192 and miR-205, were correlated with the degree of glomerulosclerosis or tubulointerstitial scarring in IgAN, indicating that there may be a possible correlation between the levels of certain miRNAs and renal pathological features and the severity of IgAN (Wang et al. 2010a; Wang et al. 2012). In this study, we observed that the expression levels of miR-223-3p and miR-3613-3p were distinguishing among different grades of severity of IgAN. However, the discrimination among IgAN subgroups was not as significant as the discrimination between early stage IgAN and controls (both healthy subjects and non-IgAN patients).

New evidences are emerging that IgA nephropathy is an immune-mediated disease with a known antigen, galactose-deficient IgA1, which can elicit an autoantibody response and formation of immune complexes that are deposited in the mesangium (Kiryluk & Novak 2014; Pillai et al. 2014). Although miR-3613 were reported with unknown biological functions, miR-223 has been identified as an important inflammatory miRNA, maintaining the homeostasis of a wide range of processes in the immune system (Haneklaus et al. 2013; Taibi et al. 2014). Recent publications revealed that the miR-223 is up-regulated in many inflammation-related disorders including rheumatoid arthritis(Fulci et al. 2010; Shibuya et al. 2013), osteoarthritis(Okuhara et al. 2012) and inflammatory bowel disease(Fasseu et al. 2010). Bao et al. published

that miR-223 was able to inhibit cell proliferation and alleviate the inflammatory status in endothelial cells of IgAN patients (Bao et al. 2014). In the present study, the putative targets for miR-223 were mainly involved in such as positive regulation of locomotion, transmembrane transport, chemical homeostasis, and cellular response to hormone stimulus. Therefore, the distinct expression pattern of miR-223 at the early stage of IgAN provides interesting opportunities for future studies on its inflammatory pathogenesis associated with IgAN.

In summary, our study reveals the possibility of using the urine miRNAs as non-invasive molecular biomarkers for IgAN at an early stage, and lays the groundwork for further functional studies to investigate the roles of miRNAs in the pathogenesis of IgAN. Further studies with larger numbers of patients and controls, especially in different races, are urgently needed to validate the expression profiles. Moreover, further studies will be crucial to provide insight into the molecular mechanisms involved in the pathogenesis of IgAN.

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Table 1(on next page)

Demographic and clinical data of the subjects in the screening cohort and validation cohort.

Clinical data is presented as the MEAN±SEM. Scr, serum creatine; UPE, urine protein excretion; MN, membranous nephropathy; MCD, minimal change disease; HC, healthy control.

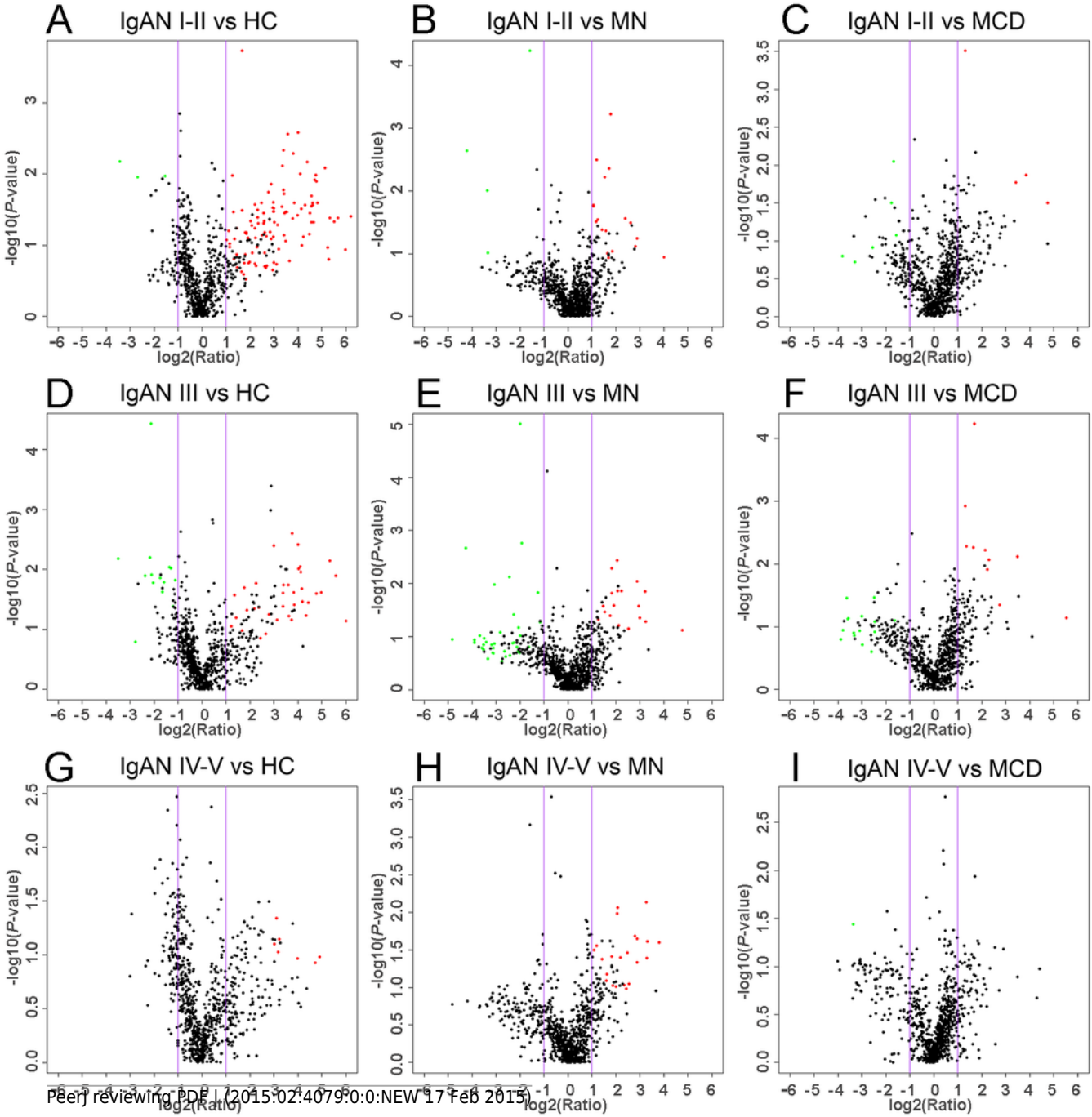
Table 1. Demographic and clinical data of the subjects in the screening cohort and validation cohort. Clinical data is presented as the MEAN±SEM.

	Cases	Gender (M/F)	Age (years)	[illegible] (μmol/L)	UPE (g/day)
the Screening Cohort					
IgAN I-II	6	3/3	27±5.3	78.7±20.22	1.21±1.78
IgAN III	6	3/3	32.3±9.3	87.3±35.56	0.82±0.75
IgAN IV-V	6	3/3	36.3±10.5	126.23±23.12	1.73±0.52
MN	4	2/2	25.8±3.1	61.63±19.50	4.17±1.66
MCD	4	2/2	33.3±8.5	82.58±25.63	8.15±2.22
HC	6	3/3	31.2±8.4	69.14±10.25	0.008±0.003
the Validation Cohort					
IgAN I-II	29	18/11	31.6±10.3	82.2±21.31	1.40±1.58
IgAN III	52	34/18	33.3±9.7	87.18±21.42	1.37±1.08
IgAN IV-V	21	15/6	36±8.8	161.79±64.58	3.12±3.13
MN	41	24/17	35.8±9.3	69.47±17.05	4.61±2.61
MCD	27	6/21	31.6±10.9	78.08±22.12	7.01±2.86
HC	34	17/17	33.1±7.9	71.0±11.89	0.015±0.013

1

Volcano plots of miRNA expression determined by miRNA microarray analysis.

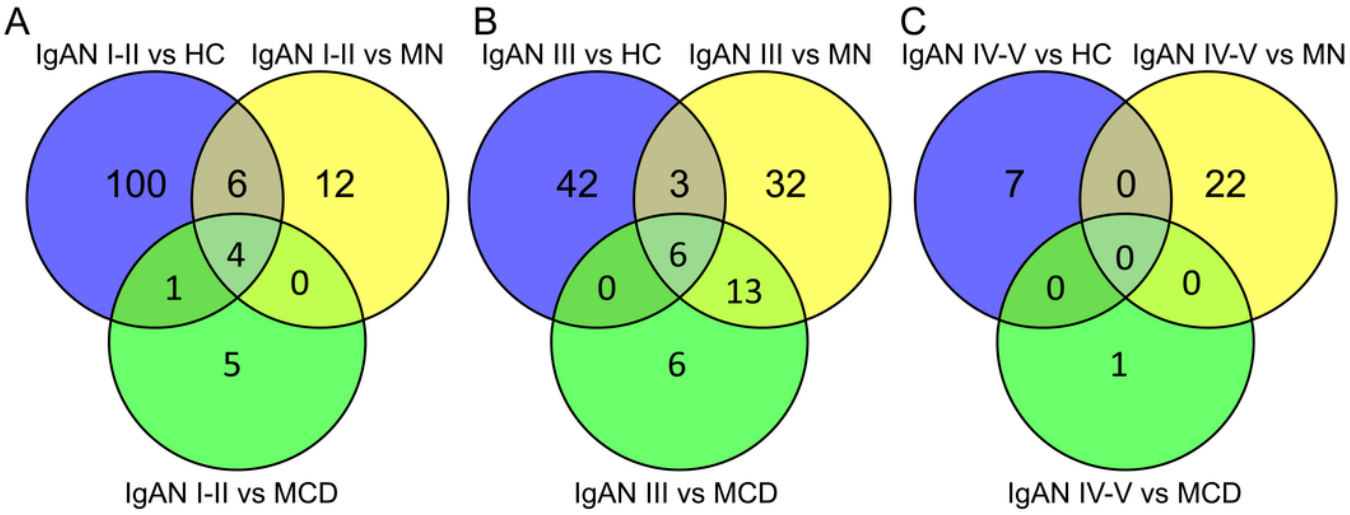
Red and green dots represent the number of miRNAs that were significantly up-regulated and down-regulated, respectively, and black dots represent a lack of differential expression. The threshold of statistically significant difference was set at $P<0.05$ and FC>2.



2

Overlapping relationship of the differentially expressed miRNAs.

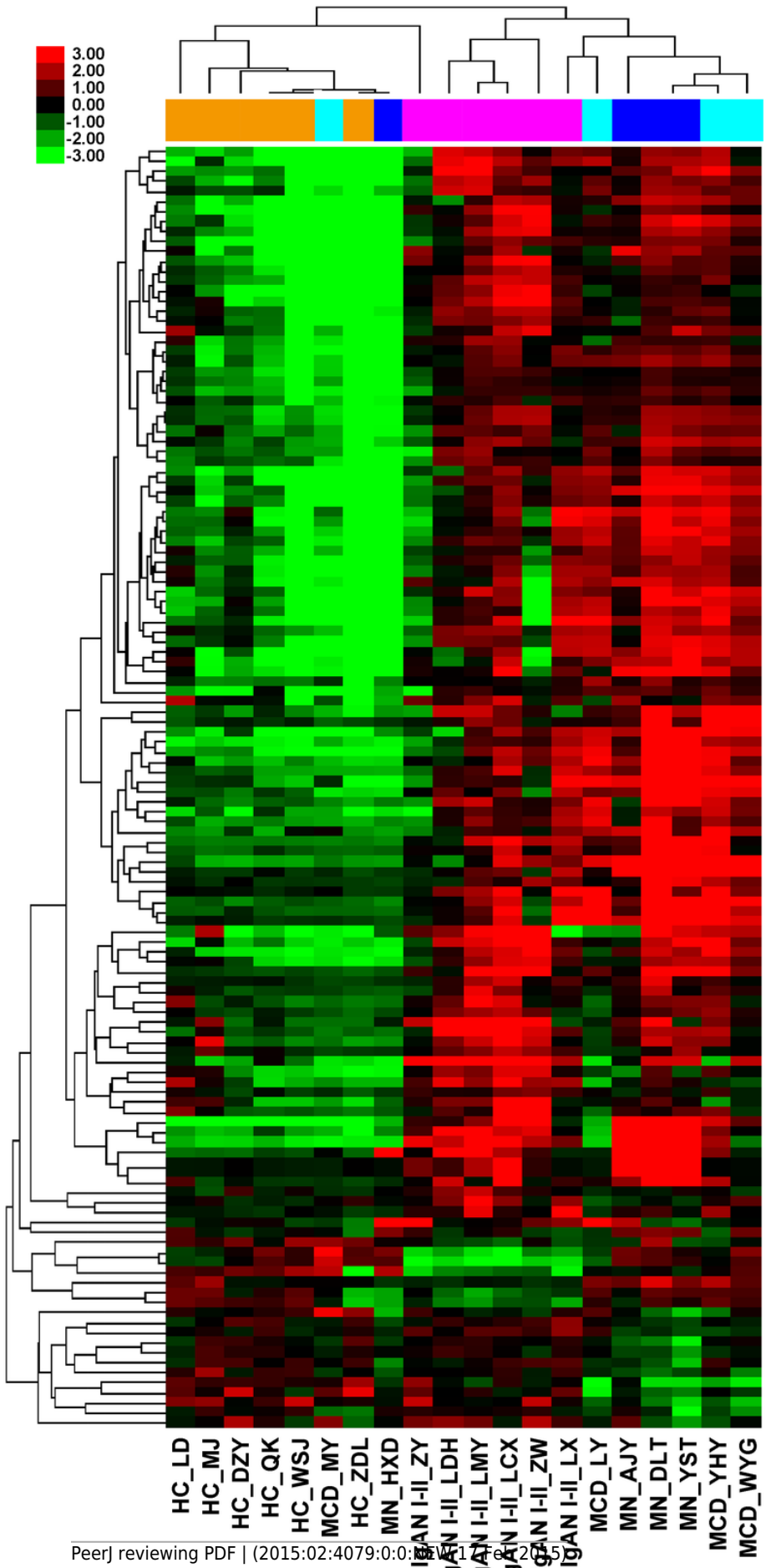
The miRNAs in the centre of the Venn diagram were (A) miR-223-3p, miR-629-5p miR-3613-3p and miR-4668-5p; (B) miR-150-5p, miR-572, miR-371b-5p, miR-3613-3p, miR-4668-5p and miR-6750-5p.



3

Supervised hierarchical clustering of IgAN patients and control subjects.

The heat map showed the separation of IgAN I-II patients from controls, based on the 128 miRNAs according to Venn diagram analysis. Red and green represent relatively high and low miRNA expression respectively.


3.00
2.00
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0.00
-1.00
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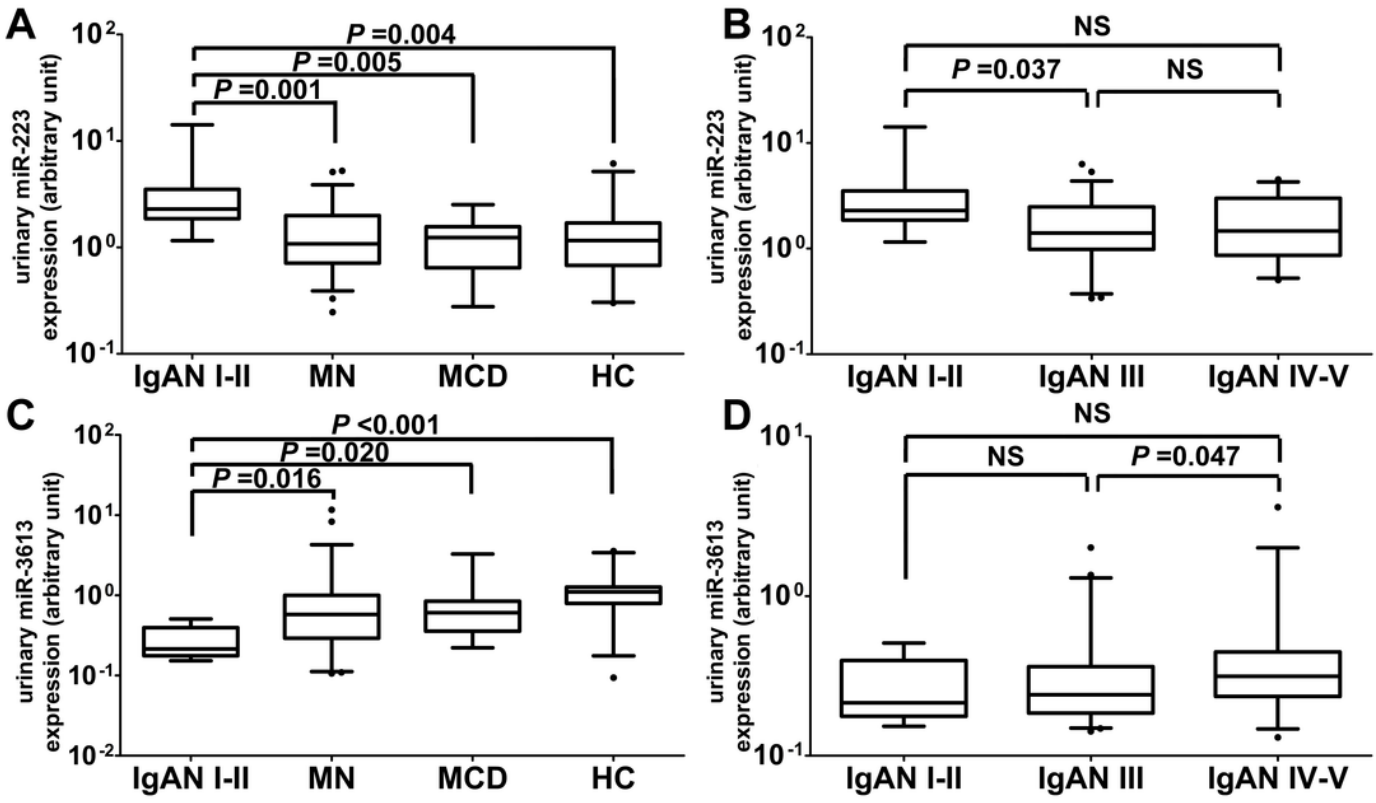
-3.00
HC_LD
HC_MJ
HC_DZY
HC_QK
HC_WSJ
MCD_MY
HC_ZDL
MN_HXD
IgAN I-II_ZY
IgAN I-II_LDH
IgAN I-II_LMY
IgAN I-II_LCX
IgAN I-II_ZW
IgAN I-II_LX
MCD_LY
MN_AJY
MN_DLT
MN_YST
MCD_YHY
MCD_WYG

4

Validation of candidate miRNAs by RT-qPCR in an independent cohort.

The Whisker-box plot depict the relative expression level of miR-223-3p and miR-3613-3p. Statistically significant differences were determined by Mann-Whitney U test. Levels are represented as ratio to the average of healthy controls.



5

GO category and KEGG Pathway enrichment analysis of predicted miRNA targets of miR-223.

P<0.05 was used as a threshold to select significant GO categories and KEGG pathways. -lgP is the negative logarithm of the P-value. The top twenty GO Biological Process and KEGG pathways were annotated.