Articles

Microarray analysis of micro-ribonucleic acid expression in primary immunoglobulin A nephropathy

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ABSTRACT

الأهداف : لاستكشاف العلاقة بين اعتلال الجلوبين المناعي (A)، الكلوي الأولي (IgAN)، والتحليل المنظم الصغير لظهور حمض ريبونيكليك (miRNA) .

الطريقة : قمنا بتحليل ظهور ملفات حمض ريبونيكليك (miRNA) في عينات كلوية من 11 (IgAN) مريضاً، و3 كمجموعة تحكم، بمركز زراعة الكلى والتنقية الدموية لـ181 مستشفى بالصين، خلال الفترة مابين مايو 2007م وحتى أكتوبر 2007م . باستعمال التحليل المنظم المصغر لحمض ريبونيكليك (miRNA) والمحتوي على التسلسلات الكاملة للإنسان البالغ وحمض ريبونيكليك (miRNA) .

النتائج : حددت الدراسة 132 ظهور لحمض ريبونيكليك (miRNA) في العينات الكلوية، منها 31 حمض ريبونيكليك (miRNA) منخفض التنظيم، و35 مرتفع التنظيم في عينات اعتلال الجلوبين المناعي الكلوي الأول . تم تأكيد النتائج بواسطة اختبارات تفاعل سلسلة الخمائر الناقلة (RT-PCR) للوقت الحقيقي .

خاتمة : قد تساعد دراستنا في إيضاح الآلية الخلوية المتعلقة في المرض ومنشأة لاعتلال الجلوبين المناعي الكلوي الأولي (IgAN) . يخدم حمض ريبونيكليك (miRNA) بشكل أساسي كعلامة تشخيص حيوية للاعتلال الكلوي للجولبين المناعي الأولي (IgAN) .

Objective: To explore the relationship between immunoglobulin A nephropathy (IgAN) and microRNA (miRNA).

Methods: We analyzed the miRNA expression profiles in renal biopsies from 11 IgAN patients and 3 controls at the Kidney Transplantation and Hemo Purification Center of 181 Hospital, China, from May to October 2007, using a mammalian miRNA microarray containing whole human mature and precursor miRNA sequences.

Results: This study identified 132 miRNAs in renal samples, of which 31 miRNAs down-regulated and 35 miRNAs up-regulated in IgAN biopsies. The chip results were confirmed by northern blot analysis and by quantitative real-time polymerase chain reaction (RT-PCR) tests.

Conclusion: Our study may help clarify the molecular mechanisms involved in the pathogenesis of IgAN, and miRNAs potentially serve as a novel diagnostic biomarker of IgAN.

Primary immunoglobulin A nephropathy (IgAN) is one of the most frequent glomerulonephritides in developed countries, which were first described by Berger and Hinglais in 1968.[1] Primary IgAN is the main cause of end-stage renal disease in patients with primary glomerular disease, which requires renal-replacement therapy.[2] Given its frequency and significance, there should be a consensus on which treatment strategy is optimal for patients with IgAN.[3] Until now, the pathogenesis of IgAN remains obscure, but new concepts have emerged during the last decade. Several work had been carried out in searching for the best prediction indices, such as circulating immunoglobulin A1 (IgA) molecules deficient in galactose (Gal),[4-5] Gal-deficient IgA1,[6,7] IgA-containing immune complexes,[8] and serum secretory IgA were detected in patients with IgAN.[9] However, none of them had been used in

clinical practice. Although specific therapy for IgAN is urgently required, the etiopathogenesis of IgAN remains unclear. Suzuki et al[10] identified the susceptibility genes and examine their roles in the pathogenesis of IgAN by a genome-wide scan using the ddY mouse model. Genome-wide linkage analysis in 30 multiplex kindreds has demonstrated linkage of IgAN to 6q22-23.[11] Although various methods had been utilized in detecting the quantities of IgA, there is still no obvious candidate genes and biomarkers for IgAN. Studies conducted during the past 3 decades have identified many abnormalities in IgAN. Recent studies also examined the roles of abnormalities in the IgA molecule itself, such as negative charge and poor glycosylation, and some of IgA receptors, in the pathogenesis of IgAN.[12-15] Therefore, a new approach is needed for the elucidation of etiopathogenesis of IgAN. MicroRNAs (miRNAs) are about 22-nucleotide, short, noncoding ribonucleic acids (RNA's) that are thought to regulate gene expression through sequence-specific base pairing with the 3'-untranslated region (3'-UTR) of target messenger ribonucleic acids (mRNA's). Hundreds of miRNAs have been identified in worms, flies, fish, frogs, mammals, and flowering plants using molecular cloning and bio-informatics prediction strategies.[16] Since the discovery of the first miRNAs lin-4 and let-7 in *Caenorhabditis elegans* in 1993,[17] at least 3420 miRNA genes in diverse organisms were identified. In recent years, several articles have been published showing a probable link between miRNAs and cancer,[18,19] fragile X syndrome,[20] spinal muscular atrophy,[21] DiGeorge syndrome,[22] and systemic lupus erythematosus.[23] MicroRNAs have also been shown to regulate insulin secretion.[24] These studies represent the beginning links between miRNAs and human disease. Further investigations are likely to reveal the involvement of additional miRNAs and their targets in simple and complex genetic diseases. Understanding the mechanisms that contribute to primary IgAN, and find biomarkers to anticipate primary IgAN will be of great value for the development of improved nephropathy treatment strategies. Until now, there is no report of primary IgAN from the aspect of miRNAs. In our study, we compared the levels of miRNA expression in renal biopsies of primary IgAN between the normal samples, and try to reveal the relation between miRNA and primary IgAN.

Methods. We studied the biopsies of 11 IgAN patients at the Kidney Transplantation and Hemo Purification Center of 181 Hospital, China during the period May to October 2007. There were 8 men and 3 women aged 16-57 years (mean: 29 years). The patients' biopsies had shown 18 glomeruli on average, based on light microscopic findings, and slight-
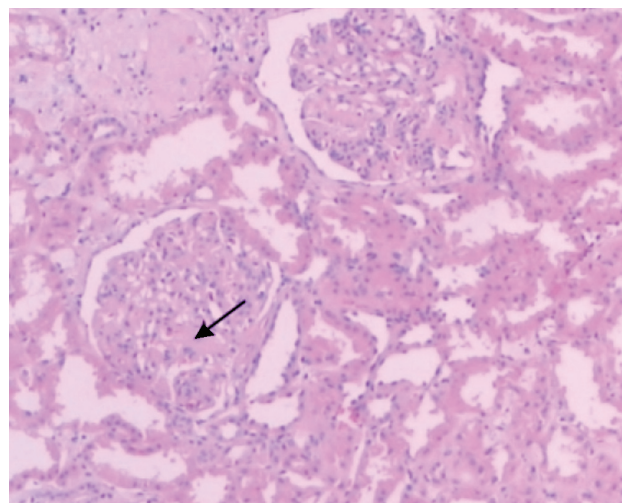

Figure 1 - Light microscopic examination for immunoglobulin A nephropathy (IgAN) biopsy (Hematoxylin and eosin stain, x400).

midrange mesangial proliferative glomerulonephritis (MesPGN) (**Figure 1**) according to Lee et al[25] grading system. There were no glomerular sclerosis (GS), glomerular atrophy, and sacculus proprius accretion. The interstitial inflammation and fibrosis were not obvious under light microscopic. Based on immunofluorescence microscopic observations, all samples exhibited more dominant mesangial deposits of IgA than of IgG and IgM with complement component 3 (c3) weakly positive, protein subunit of complement I and complement component 4 (c4) negative. Renal biopsies were performed based on clinical indication with ultrasound guidance, using the BIOPTYo instrument. The samples were renal cortex, obtained from aspiration-needle biopsy. All patients' diagnosis was confirmed by histology tests. The 3 samples for control group were renal cortex taken from resection operation of renal tumor, which is located far from the tumor tissue, and tissue structures were normal through light microscope checking. A written informed consent was obtained from all subjects or their guardians. The use of biopsy material for further studies beyond routine diagnosis was approved by the local ethics committee. This study was also performed according to the guidelines of Chongqing Medical University, which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects.

Histological analysis. Biopsy material was immediately fixed in 10% phosphate-buffered paraformaldehyde, and stored at 4°C. After fixation, biopsies dehydrated through ascending ethanol series were embedded in EPON 812. The serial semi-thin sections (0.5 μm thick) were cut on a Reichert Ultracut

Table 1 - Reverse-transcribe and QRT-PCR primers.

Gene name	QRT-PCR primers	Anneal temperature (°C)	Product length (bp)
U6	F:5' GCTTCGGCAGCACATATACTAAAAT 3' R:5' CGCTTCACGAATTTGCGTGTCAT 3'	60	89
hsa-miR-637	F:5' ACTGGGGGCTTTCGGG R:5'AGTGCGTGTCGTGGAGTC3'	60	58
hsa-miR-492	F:5' GAGGACCTGCGGGACAA3' R:5'AGTGCGTGTCGTGGAGTC3'	60	62

Bp - base pair, QRT-PCR - quantitative reverse transcriptase polymerase chain reaction

Emicrotome. Resin was removed by treatment of sections with sodium methoxide prior to rehydration and immuno-staining as previously described.

Preparation of renal tissue samples. Aspiration-needle biopsy or renal cortex pieces (<0.3 x 0.3 x 0.3 mm^3) obtained after nephrectomizing was immediately washed by 0.9% NaCl (RNase-free), quickly dipped in RNase Inhibitor (Epicentre, Mexico, USA) according to the manufacturer's instructions. After storing in 4°C overnight, the depressor was removed from the biopsies, and the biopsies were stored in -80°C for further test.

MicroRNA isolation. Total RNA was extracted from renal cortex pieces using Trizol (Invitrogen, California, USA) according to the manufacturer's instructions. The concentration and quality of RNA were measured by the UV absorbance at 260 nm and 280 nm (A260/280), and checked by gel electrophoresis. All RNA was mixed in each sample group, and used for miRNA isolation and quantitative real-time polymerase chain reaction (QRT-PCR) verification. MicroRNA isolation was carried out from the total RNA using mirVanaTM miRNA isolation kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions.

MicroRNA microarray. Locked nucleic acid modified capture probes were bought from Exiqon corporation of Denmark. MicroRNA microarray composes of 455 human miRNAs, 236 rat miRNAs, 344 mouse miRNAs, can detect all human miRNAs, rat miRNA, mouse miRNAs, and other species miRNAs in miRNA Sanger base (release 8.1). We used a novel microarray platform (miChip) that accurately and sensitively monitors the expression of miRNAs without prior need for RNA size fractionation and/or amplification and that can discriminate among closely related miRNA family members.

MicroRNA microarray analysis. MicroRNA was labeled by miRCURYTM Array Labelling kit (Cat #208032, Exiqon, Denmark) then concentrated the labeled sample by RNeasy Mini Kit (Cat #74104,

Qiagen, Germany) according to the manual. Hy3™ fluorescent label was used for dyeing. The hybridization was carried out according to the manufacturers instructions, 635 nm laser was used to scan the slide using the Genepix 4000B, and data analyzed by Genepix Pro 6.0. Hybridization was carried out twice on 2 different days.

Data analysis. Signal intensities for each spot were analyzed and calculated by the Image Quant 5.0 (Amersham Pharmacia Biotech Ltd., USA and Array vision 6.0 (Imaging Research Ltd., USA. Signal intensities for each spot were scanned and calculated by subtracting local background (based on the median intensity of the area surrounding each spot) from total intensities. An average value of the 2 spot replicates of each miRNA was generated after data transformation (to convert any negative value to 0.01), normalization was performed by using a per-chip 50th percentile method that normalizes each chip on its median, allowing comparison among chips. To highlight miRNAs that characterize each group, a per-gene on median normalization was performed, which normalizes the expression of every miRNA on its median among samples.

Quantitative real-time PCR verification of miRNA microarray results. Ribonucleic acid was reverse transcribed to complementary DNA with gene-specific primers. Quantitative real-time polymerase chain reaction primers are listed in **Table 1**, and cycle parameters for the PCR reaction was 95°C for 15 minutes, followed by 40 cycles of a denaturing step at 95°C for 10 seconds, and an annealing/extension step at 60°C for 60 seconds. All reactions were run in triplicate. The relative amount of each miRNA to U6 RNA was described by using the equation $2^{-\Delta Ct}$, where $\Delta Ct=(Ct_{miRNA}-Ct_{U6})$. The miRNAs analyses were hsa-miR-637 and hsa-miR-492.

Results. The quantity and quality of the RNA samples in IgAN and normal control (NC) group

Table 2 - Sixty-five MiRNAs with significant different expression levels detected in both immunoglobulin A nephropathy (IgAN) and normal control (NC) groups. Normalized hybridization signal of miRNAs identified in the microarray analysis are listed in this table.

miRNA name	NC calibrated-sARVOL	IgAN calibrated-sARVOL	IgAN/NC ratio[†]
34 microRNAs down-regulated			
hsa-miR-150_MM1	1.049	0.062	0.059
hsa-miR-615	0.603	0.043	0.071
hsa-miR-296	1.252	0.127	0.101
hsa-miR-133a_MM1	0.215	0.022	0.102
hsa-miR-637	0.682	0.074	0.108
hsa-miR-133a-133b	0.227	0.026	0.115
hsa-miR-611	0.166	0.019	0.116
hsa-miR-557	3.825	0.504	0.132
hsa-miR-365	0.247	0.033	0.134
hsa-miR-99a	0.091	0.013	0.146
hsa-miR-663	12.857	2.409	0.187
hsa-miR-202_MM1	1.451	0.297	0.205
hsa-miR-518b	0.177	0.040	0.226
hsa-miR-346	0.409	0.094	0.230
hsa-miR-550	0.042	0.010	0.232
hsa-miR-30d	0.179	0.045	0.251
hsa-miR-596	0.060	0.015	0.255
hsa-miR-642	0.718	0.189	0.263
hsa-miR-345_MM1	0.242	0.067	0.277
hsa-miR-484	0.264	0.074	0.281
hsa-miR-552	0.021	0.006	0.286
hsa-miR-324-3p	0.701	0.201	0.287
hsa-miR-585	0.059	0.018	0.299
hsa-miR-654	0.209	0.063	0.300
hsa-miR-223	0.089	0.027	0.303
hsa-miR-635	0.030	0.009	0.312
hsa-miR-150	0.018	0.006	0.342
hsa-miR-625	0.222	0.090	0.405
hsa-miR-210	2.294	1.007	0.439
hsa-let-7d_MM1	0.066	0.031	0.473
hsa-miR-486	0.128	0.061	0.479
34 microRNA's up-regulated			
hsa-miR-659	2.969	5.959	2.007
hsa-miR-628	0.526	1.071	2.037
hsa-miR-648	0.251	0.515	2.052
hsa-miR-483	0.182	0.382	2.100
hsa-miR-198	0.956	2.076	2.171
hsa-miR-197_MM2	0.213	0.486	2.282
hsa-miR-518c*	3.540	8.433	2.383
hsa-miR-526b	0.128	0.311	2.423
hsa-miR-23a	0.028	0.068	2.474
hsa-miR-302b*_MM1	1.306	3.314	2.538
hsa-miR-600	0.010	0.028	2.780
hsa-miR-657	0.010	0.028	2.984
hsa-let-7a	0.057	0.187	3.310
hsa-miR-185	1.399	4.809	3.438
hsa-miR-494	4.539	16.183	3.565
hsa-miR-512-5p	0.912	3.315	3.634
hsa-miR-612	12.344	48.593	3.937
hsa-miR-608	0.012	0.052	4.202
hsa-miR-658	1.257	5.532	4.402
hsa-miR-433	0.008	0.038	4.758
hsa-miR-134	0.043	0.221	5.166
hsa-miR-325_MM2	0.007	0.038	5.691
hsa-miR-513	8.233	46.918	5.699
hsa-miR-320	3.866	22.835	5.907
hsa-miR-601	0.134	0.917	6.853
hsa-miR-324-5p	0.012	0.081	6.865
hsa-miR-15b_MM1	0.003	0.026	7.433
hsa-miR-208	0.002	0.016	7.943
hsa-miR-622	0.005	0.040	8.109
hsa-miR-30a-5p	0.025	0.248	9.994
hsa-miR-195	0.005	0.050	10.289
hsa-miR-130b	0.010	0.125	13.093
hsa-miR-662	0.042	0.639	15.170
hsa-miR-124a	0.001	0.044	50.968

miRNAs are arranged according to the IgAN/NC ratio. †if the ratio was less than 2 or less than 0.5, the difference was significant. Calibrated-sARVOL - normalized fluorescence intensity with background subtracted

Table 3 - Sixty-six miRNAs without significant different expression levels detected valid expression in both immunoglobulin A nephropathy (IgAN)and normal control (NC) groups. Normalized hybridization signal of miRNAs identified in the microarray analysis are listed in this table.

miRNA Name	NC calibrated-sARVOL	IgAN calibrated-sARVOL	IgAN/NC ratio[†]
hsa-miR-452	1.141	0.580	0.508
hsa-miR-381_MM1	0.212	0.109	0.512
hsa-miR-125a_MM1	0.185	0.100	0.543
hsa-miR-769-.3p	8.667	4.760	0.549
hsa-miR-125b	0.086	0.047	0.550
hsa-miR-510	0.312	0.175	0.562
hsa-miR-629	0.099	0.057	0.579
hsa-miR-645	0.035	0.020	0.589
hsa-miR-526c	0.388	0.229	0.590
hsa-miR-129	1.663	1.000	0.601
hsa-miR-373*	1.875	1.135	0.605
hsa-miR-519d	0.054	0.033	0.617
hsa-miR-370	0.244	0.151	0.619
hsa-miR-100_MM2	0.028	0.018	0.652
hsa-miR-551a	0.117	0.078	0.663
hsa-miR-572	8.971	6.095	0.679
hsa-miR-525*-524	0.018	0.013	0.706
hsa-miR-107	0.161	0.117	0.723
hsa-miR-527	0.464	0.363	0.781
hsa-miR-500	0.711	0.559	0.787
hsa-miR-602	5.027	4.094	0.814
hsa-miR-214	0.331	0.276	0.835
hsa-miR-198_MM2	2.871	2.430	0.846
hsa-miR-451	0.021	0.018	0.853
hsa-miR-520d*	0.029	0.026	0.895
hsa-miR-524*	0.241	0.227	0.942
hsa-miR-381	0.186	0.176	0.948
hsa-miR-183_MM1	0.080	0.078	0.974
hsa-miR-617	0.673	0.659	0.979
hsa-miR-92b_MM2	0.389	0.404	1.037
hsa-miR-202	1.252	1.350	1.079
hsa-miR-103	0.115	0.127	1.109
hsa-let-7b	0.758	0.846	1.116
hsa-miR-518f*-526a	1.247	1.458	1.169
hsa-miR-382	0.233	0.276	1.186
hsa-let-7c	1.353	1.615	1.193
hsa-miR-423	0.082	0.099	1.207
hsa-miR-197	0.086	0.105	1.217
hsa-miR-525	0.745	0.921	1.236
hsa-miR-17-3p_MM1	0.220	0.272	1.237
hsa-miR-520a*	0.055	0.069	1.255
hsa-miR-519e*	0.529	0.668	1.263
hsa-miR-638	3.155	3.986	1.264
hsa-miR-630	1.122	1.486	1.324
hsa-miR-671	10.823	14.923	1.379
hsa-miR-204_MM1	0.018	0.025	1.379
hsa-miR-361	0.354	0.490	1.382
hsa-miR-142-5p	0.021	0.029	1.400
hsa-miR-498	6.285	9.289	1.478
hsa-miR-583	2.477	3.680	1.485
hsa-miR-326	0.183	0.306	1.668
hsa-miR-197_MM1	0.133	0.223	1.675
hsa-miR-634	0.197	0.331	1.682
hsa-miR-490	0.175	0.297	1.702
hsa-miR-503	6.579	11.351	1.725
hsa-miR-125a	0.117	0.205	1.763
hsa-let-7e	0.124	0.221	1.781
hsa-miR-184	0.072	0.129	1.784
hsa-miR-492	6.066	10.835	1.786
hsa-miR-200c	0.013	0.023	1.817
hsa-miR-516-5p	0.173	0.320	1.848
hsa-miR-584	1.153	2.132	1.849
hsa-miR-330_MM1	0.037	0.069	1.859
hsa-miR-623	6.407	12.416	1.938
hsa-let-7a_MM1	0.048	0.095	1.989
hsa-miR-575	0.008	0.017	1.997

miRNAs are arranged according to miRNA names. †if the ratio was less than 2 or less than 0.5, the difference was significant. Calibrated-sARVOL - normalized fluorescence intensity with background subtracted

were checked by gel electrophoresis (**Figure 2**), and absorbance at A260/280 ratio (in NC [1.85], IgAN [2]). The A260/280 ratio and gel electrophoresis results confirmed the good quality of RNA isolated. After normalization of the raw data, 131 miRNAs were detected in both groups with 65 of them differentially expressed by miRNA microarray, in which 31 miRNAs down-regulated, and 34 miRNAs up-regulated in absorbance ratio (AR) compared with the NC group (**Table 2**), while 66 miRNAs without significant different expression levels (**Table 3**). Microarray data are consistent with the QRT-PCR verification results (**Table 4**) for that the AR/NC ratio of hsa-miR-637 was 0.108, and hsa-miR-492 was 1.786 in microarray test, while in QRT-PCR test, ratio for hsa-miR-637 was 0.095, and hsa-miR-492 was 1.659, which was close.

Discussion. In the present study, we comprehensively isolated and analyzed miRNAs in the patients' tissue of primary IgAN and normal control, using miRNA

Figure 2 - Gel electrophoresis check of pooled total RNA from immunoglobulin A nephropathy (IgAN) and normal control (NC) samples.

microarray analysis. This study identified 132 miRNAs in renal samples, of which 31 miRNAs down-regulated and 35 miRNAs up-regulated in IgAN biopsies. Immunoglobulin A neuropathy is a relatively newly recognized disease since described by Berger and Hinglais in 1968.[1] It is now generally known to be the most common form of primary glomerulonephritis throughout the world.[26-28] Though many research on IgAN's pathogenesis and methods of treatment, Batra et al[29] hypothesized that the activated systemic homing cluster of differentiation 4 -T cells may direct the aberrant systemic pIgA production observed in IgAN.[30] Praga et al[30] treat IgAN with angiotensin-converting enzyme (ACE) inhibitors, and ACE had been reported as one susceptibility genes of IgAN). Until recently, there was no effective treatment available for patients with IgAN. Primary IgAN rate was different in different gender, race and location. Generally, male have high primary IgAN rate than female, the ratio was 2:1 in Japan, and 6:1 in northern Europe and the United States, and the lower prevalence in blacks than whites, and Asians. It indicated that primary IgAN were associate closely with gene. MicroRNA is the regulator of mRNA, indirectly regulates protein expression. The link between miRNAs and some human diseases has been proven. In this study, we apply miRNA microarray chip to analyze relationship between IgAN and miRNAs, which has not been studied before. From IgAN and normal control kidney biopsies, we identified 131 miRNAs, 65 of which were found with significantly different expression levels. For these 20 miRNAs, we can predict their targets with computational target predictions such as miRanda available at http://www.microrna.org, and mirBase available at http://microrna.sanger.ac.uk/targets/v2/etc.[31] For example, the up-regulated hsa-miR-125a, 1180 hits was found in the target predicting database. The individual miRNA variation between patients may be difficult to detect with no significance, due to random changes that may exist in each patient. In our study, all RNA from each sample group was merged so that the individual
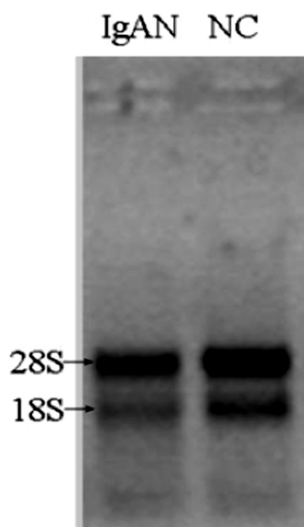
Table 4 - Quantitative reverse transcriptase-polymerase chain reaction confirmation data.

Samples	Ct_{U6}	Ct_{miRNA}	$\Delta Ct=$ $(Ct_{miRNA}-Ct_{U6})$	$Ct_{(IgAN-NC)}=$ $\Delta Ct_{IgAN}-\Delta Ct_{NC}$	$2^{-Ct(IgAN-NC)}$	IgAN/NC ratio
hsa-miR-637						0.095
NC	13.06	19.86	6.8	0	1	
IgAN	12.91	23.11	10.2	3.4	0.095	
hsa-miR-492						1.659
NC	13.06	16.93	3.87	0	1	
IgAN	12.91	16.05	3.14	-0.73	1.659	

Ct - Ct value, Ct is a cycle threshold, NC - normal control group, IgAN - immunoglobulin A nephropathy

difference between subjects is eliminated. The aim of this study is to reveal the relationship between IgAN and miRNAs to the public, expecting to draw other research groups' attention to this area. The lack of functional research on IgAN related miRNAs we detected, is the limitation of this study.

Taken together, we identified the 65 miRNAs differentially expressed in IgAN whose expression profiling may provide a useful clue for the pathophysiology research of IgAN. Our work indicates that miRNAs are potential diagnosis biomarkers and probable factors involved in the pathogenesis of IgAN. Further investigation is needed to clarify the roles of identified miRNAs in the pathogenesis of IgAN. Our study of miRNAs may lead to finding novel methods to diagnosis, treat, and prevent IgAN, and to provide a novel researching method for it.

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