MicroRNAs as a therapeutic target in IgA nephropathy in Indian population

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Abstract. Immunoglobulin A nephropathy (IgAN) is the most frequent glomerular disease with rapid development to end stage renal disease, requiring renal replacement therapy. Genome-wide studies suggest geographical variations in genetic susceptibility to IgAN and disease progression. Specific 'candidate genes' were indicated to correlate with different functions that are involved in the pathogenesis of renal conditions. MicroRNAs (miRNAs/miRs) have a major role in mRNA degradation or translation repression, thereby regulating the expression of their target proteins. Previously, a small number of miRNAs were reported to have direct associations with IgAN. In the present study, new miRNAs linked to IgAN were identified in the Indian population. The miRNA was isolated from kidney biopsies of patients with IgAN (n=6) and healthy control tissue from patients with renal cell carcinoma (n=6). The sequencing results indicated that the miRNA percentage acquired from controls and patients with IgAN was 5.61 and 4.35%, respectively. From the results, 10 upregulated and 15 downregulated miRNAs were identified. Of the 25 differentially expressed miRNAs (DEMs), miR-181a-5p, miR-28-3p, let-7g-5p, miR-92a-3p and miR-30c-5p were not reported previously. Furthermore, Kyoto Encyclopedia of Genes and Genomes and Gene Ontology analyses suggested that the target genes of the DEMs were mainly enriched in pathways such as cancer, ErbB signalling, proteoglycans in cancer, Hippo signalling and MAPK pathways. The newly identified miRNAs may impact the behaviour of tissues or IgA deposition by regulating signalling pathways, which forms a basis for future studies aimed at improving the diagnosis and care of patients with IgAN in the Indian community.

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Abbreviations: IgAN, immunoglobulin A nephropathy; miRNA/miR, microRNA; NGS, next generation sequencing; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: immunoglobulin A nephropathy, microRNA, next generation sequencing, kidney biopsy

Introduction

Immunoglobulin A nephropathy (IgAN) is the most prevalent type of primary glomerulonephritis and the main cause of end stage renal disease (ESRD) in the world (1,2). In India, IgAN presents as nephrotic syndrome with rapid progression to ESRD, requiring renal replacement therapy (3). Experimental models suggest that surplus production of aberrantly-glycosylated IgA in mucosal-allied lymphoid tissue in the gut leads to IgA nephropathy (4,5). The four important steps in the pathogenesis of IgAN are i) mucosal IgA production by plasma cells, ii) production of under-glycosylated IgA, iii) production of immune complexes and iv) deposition of these nephritogenic immune complexes in the kidney leading to IgA nephropathy. The heterogeneous clinical features of IgAN range from asymptomatic microscopic haematuria to a rapidly progressive form of glomerulonephritis (6,7). Renal biopsy is the primary method for IgAN diagnosis and disease assessment. However, it is difficult to perform due to its invasiveness (8). Hence, there is a need to investigate the underlying mechanisms of disease development for the identification of new disease biomarkers/target genes for IgAN diagnosis that may provide an alternative to invasive methods.

MicroRNAs (miRNAs/miRs) are endogenous, small non-coding RNAs of ~18-22 nucleotides in length, which have a major role in mRNA degradation or translation repression, thereby regulating the expression of their target proteins. Several biological and molecular processes are mediated by miRNAs. Certain miRNAs regulate the target genes involved in different cellular processes, such as proliferation, differentiation, migration, signaling and apoptosis (9). The expression profiles of miRNAs are specific and vary in different cancer types, such as lung (10), liver (11), breast (12), colon (13), pancreatic (14) and renal cancer (15,16). In addition, the dysregulation of miRNAs is associated with several pathological conditions, including IgAN (17-19). Recently, a small number of miRNAs that are differentially expressed in the blood,

urine and renal tissue were identified to have a direct connection with IgAN (18,20,21). Identifying miRNAs in tissues responsible for the pathway leading to aberrant galactosylation of IgA helps to plan oral therapy that selectively inhibits the responsible miRNAs locally in the intestine without systemic serious adverse events. Studies have indicated that miRNAs are associated with a spectrum of clinical aspects in kidney disease, including fibrosis and inflammation (22,23). The association of miRNAs with IgAN progression in tissues and their role in disease pathogenesis remain largely elusive. In the present study, new miRNAs related to IgAN were discovered by sequencing miRNAs from renal biopsies. Furthermore, bioinformatics analysis was used to look into the probable functions of differentially expressed miRNAs (DEMs). A total of 25 miRNAs that were differentially expressed were found and the relationship of the majority of these miRNAs with IgAN suggests that they may act as targets or biomarkers for IgAN in the Indian population. The objectives of the present study were to assess miRNAs as potential disease markers and further explore miRNA-based targeted therapeutic strategies for IgAN.

Materials and methods

Clinical sample collection. The present study was a single-centre hospital-based case-control, retrospective study, which was conducted at AIG (Mayo Clinic Care Network) Hospital (Hyderabad, India) from February 2021 to April 2022, that included the kidney biopsy blocks of patients with IgAN (n=6) and control tissue (n=6) from patients with renal cell carcinoma ('healthy' tissue). The samples were confirmed by in-house pathologists. After obtaining informed consent from the patients, biopsy samples were collected from the surgical wards. The inclusion criteria for patients with IgAN were as follows: i) IgAN was confirmed by renal biopsy; ii) normal renal function (blood urea nitrogen, blood creatinine); iii) age ≥25 years; iv) no previous hormone, immunosuppressant or kidney transplantation treatments; and v) the patients did not have any secondary IgAN, such as lupus nephritis, purpura nephritis or hepatitis B-related nephritis. The exclusion criteria for healthy participants were patients with i) chronic diseases, such as coronary heart disease, hypertension, acute and chronic cerebrovascular disease or diabetes; ii) infectious diseases and fever; iii) mental illnesses; and iv) any metabolic syndromes. The research study was approved by the Institutional Research and Ethics Committee of AIG Hospital (AHF/AIGH-IRB: 02/47/2021) and written informed consent was obtained from all patients included in the present study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The clinical features of patients with IgAN after diagnosis by experienced pathologists are provided in Table I.

Tissue collection and miRNA isolation. Formalin-fixed paraffin-embedded (FFPE) blocks of the biopsy specimens were collected. Sections (3-5 mm) were cut from each block and a single section from each block was mounted on previously prepared poly-L-lysine-coated slides. The sections were then subjected to haematoxylin and eosin staining according to a standard protocol. Images of the stained slides were acquired using a bright-field microscope (DM 2000; Leica Microsystems). The pathological analysis of samples was performed by evaluating both macroscopic and microscopic features of kidney tissues. The slides were reviewed by experienced in-house pathologists in a blinded manner.

Table I. Demographic and baseline clinical data of the healthy participants and patients with IgAN.

Characteristic	IgAN (n=6)	Healthy controls (n=6)
Sex, male/female	5:1	5:1
Age, years	51.2±7.49	59±5.91

Values are presented as n or the mean ± SD. IgAN, IgA nephropathy.

Frozen kidney biopsy specimens from six patients with IgAN and six healthy control samples were selected for miR transcriptomic profiling by next generation sequencing (NGS). miRNA was extracted and purified using the miRNeasy FFPE Kit (cat. no. 217504; Qiagen GmbH) following the manufacturer's protocol. Isolated miRNAs were quantified with the Qubit microRNA Assay Kit (cat. no. Q32880; Qiagen GmbH) and were included in the further analysis.

Library preparation for small RNA sequencing (RNA-seq). Small RNA library preparation was performed using the CleanTag™ Small RNA Library Preparation Kit (cat. no. L-3206; TriLink Biotechnologies) according to the manufacturer's protocol. In brief, isolated miRNA molecules were ligated with a 3' RNA adapter, followed by ligation with a 5' adapter. The cDNA was prepared at 50˚C for 1 h and amplified. PCR was performed using the following thermocycling conditions: Initial denaturation at 98˚C for 30 sec; followed by 18 cycles of 98˚C for 10 sec, 60˚C for 30 sec and 72˚C for 15 sec; followed by a final extension at 72˚C for 10 min. Purified library products were evaluated using the High Sensitivity DNA Chip for Agilent 2100 Tape Station (Agilent Technologies, Inc.) and the samples were again quantified by using the Qubit microRNA Assay Kit, followed by NGS on the ION S5 Torrent (Thermo Fisher Scientific, Inc.) at 1x50 base pairs.

miRNA expression analyses

Quality control and reads mapping to the reference genome. BAM files of raw reads were processed and high-quality reads were obtained. The upstream and downstream analyses were mainly based on the clean and high-quality reads. The sequences of miRNA reads were mapped to the reference Human genome (hg38) by Bowtie2 (v2.2) using Partek Flow Genomic Analysis Software (https://www.partek.com/partek-flow/).

Known miRNA alignment and novel miRNA prediction. miRBase version 22 (https://www.mirbase.org/) was used as a reference. The Partek Flow Genomic Analysis Software was used to compare the miRNA expression between healthy participants and patients with IgAN and to calculate the P-value. These softwares were used to predict novel miRNA as well as helped to identify novel miR counts, base bias on either the first position or on each position of all novel miRs.

Table II. Differentially expressed miRNAs and their respective associated upregulated and downregulated genes.

A, Significant differentially expressed genes summary (Test vs. Control)

MiRNA ID	FDR step up	Ratio	Fold change	P-value
hsa-miR-21-5p	0.0008	6.894	6.89	0.00002
hsa-miR-10a-5p	0.4835	0.650	-1.54	0.2805
hsa-miR-146b-5p	0.0346	3.494	3.49	0.0037
hsa-miR-29c-3p	0.8341	0.810	-1.23	0.7148
hsa-miR-192-5p	0.6392	1.255	1.26	0.5015
hsa-miR-204-3p	0.0530	0.269	-3.71	0.0114
hsa-miR-92a-3p	0.1016	1.992	1.99	0.0297
hsa-miR-328-3p	0.0530	0.303	-3.30	0.0113
hsa-miR-146a-5p	0.4055	1.827	1.83	0.2059
hsa-miR-155-5p	0.0501	2.786	2.79	0.0092
hsa-let-7g-5p	0.2391	2.791	2.79	0.1067
hsa-miR-184	0.1211	0.241	-4.15	0.0404
hsa-miR-28-3p	0.1211	1.803	1.80	0.0410
hsa-miR-320c	0.0601	0.217	-4.62	0.0148
hsa-miR-486-5p	0.0189	0.150	-6.65	0.0017
hsa-miR-139-3p	0.0110	0.416	-2.40	0.0006
hsa-miR-99a-5p	0.0110	0.329	-3.04	0.0008
hsa-miR-1307-3p	0.8103	0.854	-1.17	0.6732
hsa-miR-30c-2-3p	0.0413	0.433	-2.31	0.0057
hsa-miR-30c-5p	0.1130	2.470	2.47	0.0348
hsa-miR-100-5p	0.0815	0.457	-2.19	0.0213
hsa-miR-320b	0.0501	0.272	-3.67	0.0085
hsa-miR-181a-5p	0.1390	1.750	1.75	0.0513
hsa-miR-151a-3p	0.1003	0.505	-1.98	0.0278
hsa-miR-127-3p	0.0110	0.159	-6.29	0.0007

B, Top 10 upregulated miRNAs in small RNA sequencing analysis

MiRNA ID	FDR step up	Ratio	Fold change	P-value
hsa-miR-21-5p	0.0008	6.894	6.89	0.00002
hsa-miR-146b-5p	0.0346	3.494	3.49	0.0037
hsa-miR-192-5p	0.6392	1.255	1.26	0.5015
hsa-miR-181a-5p	0.1390	1.750	1.75	0.0513
hsa-miR-92a-3p	0.1016	1.992	1.99	0.0297
hsa-miR-146a-5p	0.4055	1.827	1.83	0.2059
hsa-miR-155-5p	0.0501	2.786	2.79	0.0092
hsa-let-7g-5p	0.2391	2.791	2.79	0.1067
hsa-miR-28-3p	0.1211	1.803	1.80	0.0410
hsa-miR-30c-5p	0.1130	2.470	2.47	0.0348

Table II. Continued.

C, Top 15 downregulated miRNAs in small RNA sequencing analysis

MiRNA ID	FDR step up	Ratio	Fold change	P-value
hsa-miR-10a-5p	0.4835	0.650	-1.54	0.2805
hsa-miR-320c	0.0601	0.217	-4.62	0.0148
hsa-miR-486-5p	0.0189	0.150	-6.65	0.0017
hsa-miR-139-3p	0.0110	0.416	-2.40	0.0006
hsa-miR-99a-5p	0.0110	0.329	-3.04	0.0008
hsa-miR-328-3p	0.0530	0.303	-3.30	0.0113
hsa-miR-1307-3p	0.8103	0.854	-1.17	0.6732
hsa-miR-30c-2-3p	0.0413	0.433	-2.31	0.0057
hsa-miR-100-5p	0.0815	0.457	-2.19	0.0213
hsa-miR-320b	0.0501	0.272	-3.67	0.0085
hsa-miR-29c-3p	0.8341	0.810	-1.23	0.7148
hsa-miR-184	0.1211	0.241	-4.15	0.0404
hsa-miR-151a-3p	0.1003	0.505	-1.98	0.0278
hsa-miR-204-3p	0.0530	0.269	-3.71	0.0114
hsa-miR-127-3p	0.0110	0.159	-6.29	0.0007

Significance was assumed if P≤0.05 and Log2Fc ≥2 and ≤-2. MiRNA/miR, microRNA; FDR, false discovery rate.

Quantification of miRNA. The miR read counts were normalized as transcript per million (TPM) based on the following formula: Normalized expression=mapped read count/total reads x10^6.

Differential expression of miRNA. The uniquely mapped reads were then subjected to differential gene expression (DGE) analysis using DeSeq2 in Partek Flow Genomic Analysis software. The Benjamini and Hochberg's false discovery rate (FDR) was used to adjust the P-values. In the expression profile, statistically significant differences of genes were considered with a fold change (FC) ≥±2 and P<0.05. The list of differentially expressed genes is provided in Table II for the respective comparison. P<0.05 was considered the significant threshold for the signal transduction and disease pathways. Furthermore, the variance of gene expression patterns between the test and healthy samples was assessed using principal component analysis (PCA). Specifically, a three-component PCA analysis and visualization were conducted with default parameters using Partek Flow Genomic Analysis Software.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of genes encoding the DEMs. The most overrepresented GO terms and KEGG pathways that were closely linked to the DEMs were highlighted using KEGG and GO functional annotations. The KEGG Database served as the basis for the KEGG Pathways study (KEGG enrichment pathway database; http://www.genome.jp/.). The Target Scan (https://www.targetscan.org/vert_80) and miRDB (http://www.mirdb.org/) tools were used to acquire the signal transduction and disease pathway

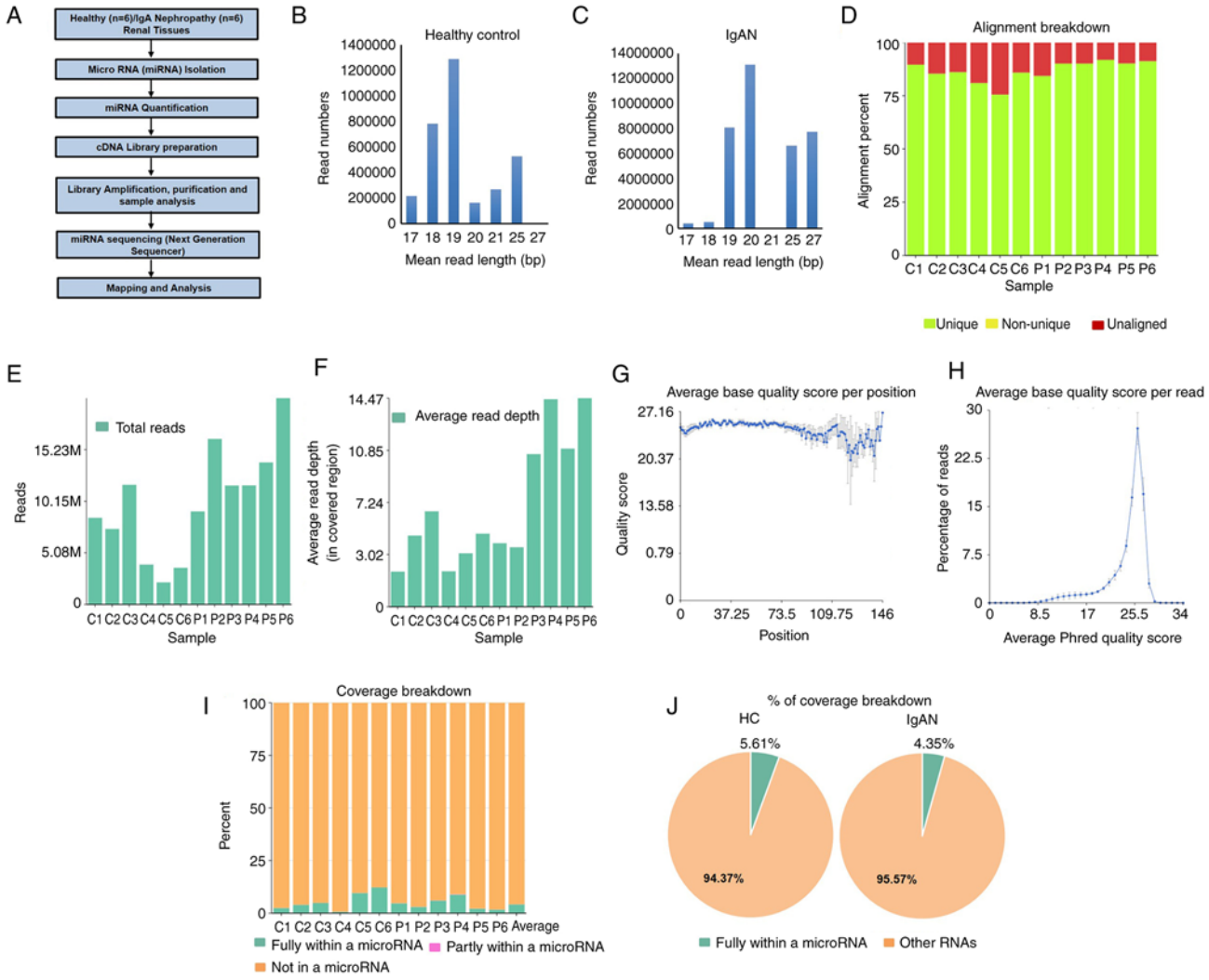

Figure 1. miRNA sequencing analysis in healthy participants and IgAN patients. (A) The workflow of the miRNA sequencing experiment. (B and C) Read length distributions of miRNA in (B) healthy participants and (C) patients with IgAN. (D-H) Alignment percentage in each sample along with (D) alignment breakdown, (E) total read count, (F) coverage read counts, (G) average base quality score per position and (H) per reads. (I and J) Quantification of coverage breakdown in both healthy participants (C1, C2….C6) and patients with IgAN (P1, P2…P6). miRNA, microRNA; IgAN, immunoglobulin A nephropathy; HC, healthy controls.

annotation data for the potential target genes. A Fisher's exact test was used to determine the P-value and a significant threshold of P<0.05 was used to evaluate the statistical significance of the signal transduction and disease pathways to the background. Cluster Profiler (Bioconductor) was used for the GO functional analysis to offer molecular function (MF), biological process (BP) and cellular component (CC) annotation for potential target genes (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html.). A statistically significant difference in the level of GO annotation enrichment was defined as P<0.05.

Regulatory network construction of miRNA-target genes. To recognize the function of miRs in the context of regulatory interactions between miRNAs and their target genes, the regulatory network of miRNA-target genes was constructed using CyTransFinder (Cytoscape3.x plugin) (24). The combined scores >0.4 were selected to construct the network and Cytoscape (version 3.6.1) software was used to further analyse the interactive miRNA-target gene network.

Statistical and bioinformatics analysis. Partek Flow Genomics Analysis Software (Build version 9.0.20.0417) was used for the quantification and statistical analysis. DGE analysis was performed using one-way ANOVA followed by with Dunnett's post-hoc test. P≤0.05 and Log2FC ≥2 was considered to indicate differentially-expressed genes. Data were further processed for GO enrichment analysis with Cluster Profiler and KEGG pathway analysis with the KEGG database. The scatter plots for gene expression and heat map were constructed using Partek Flow Analysis Software.

Results

miRNA sequencing and analysis in healthy participants and patients with IgAN. To study the aberrant expression of miRNAs in the tissues of patients with IgAN, miRNAs were sequenced and the data were analyzed following the workflow presented in Fig. 1A. miRNAs isolated from the tissues of healthy participants and patients with IgAN were mainly composed of ~18-27-bp sequences (Fig. 1B and C). 19 and 20 bp nucleotides

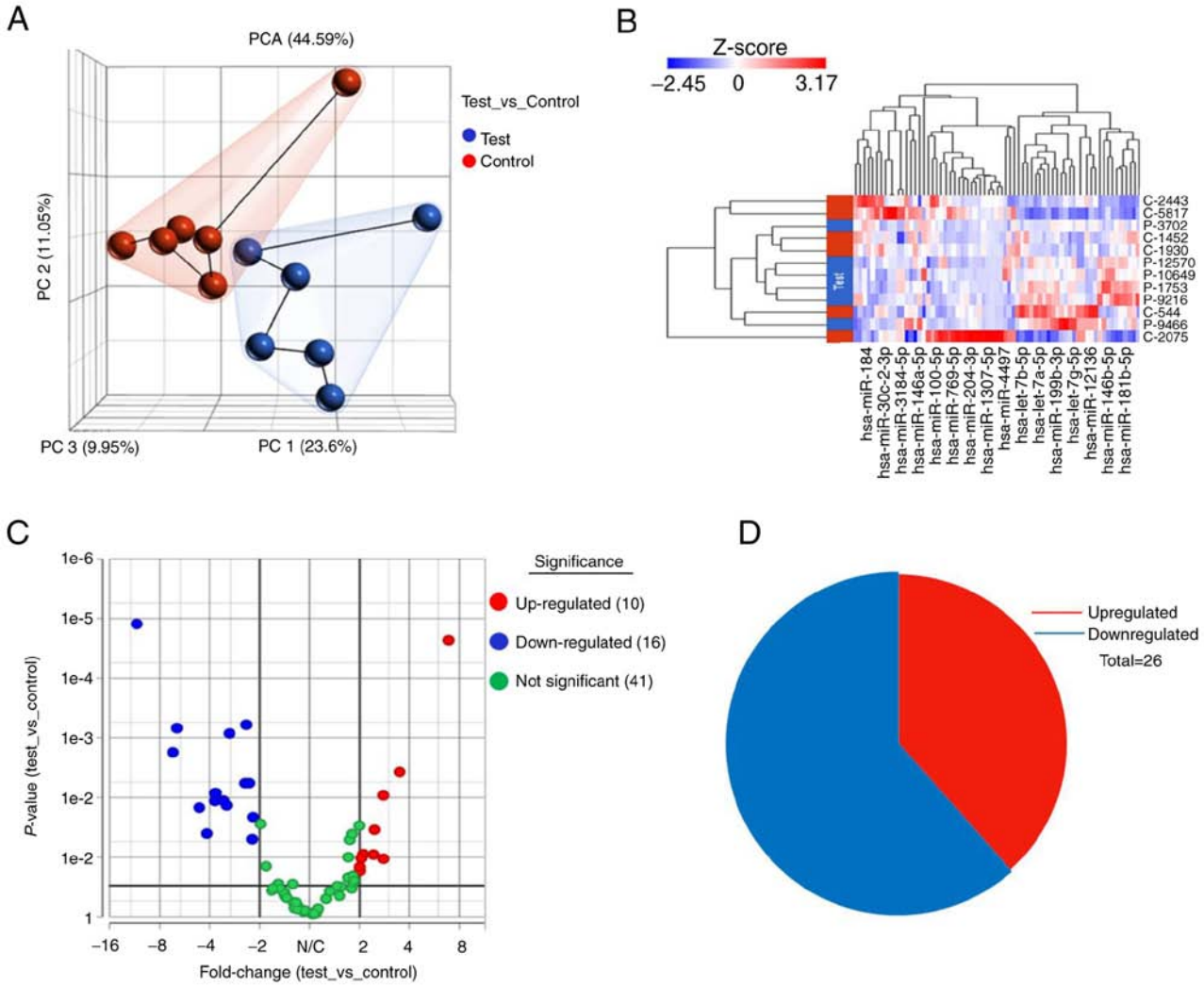

Figure 2. Differentially expressed miRNA profiles in healthy participants and patients with IgAN. (A) Scatter plot of three-dimensional PCs of the data for patients with IgAN and healthy controls. (B) Heat map of significantly differentially expressed miRNAs in healthy controls as well as in patients with IgAN. The red color indicates high expression level and blue color indicates lower expression levels (FC ≥2 or ≤0.5 and P≤0.05). (C) Volcano plot indicating numbers of upregulated and downregulated miRNAs in patients with IgAN as compared to healthy controls. Each point in the plot represents a gene and Log2-fold change and statistical significance (P≤0.05) of each gene was calculated based on differential gene expression analysis. Red points indicate significantly upregulated genes and blue points indicate significantly downregulated genes. The x-axis indicates the fold change with no significant change as the mid-point. (D) Pie chart of distributions of significantly differentially expressed upregulated and downregulated miRNAs in patients with IgAN (P1, P2…P6) compared with healthy controls (C1, C2…C6). miRNA, microRNA; IgAN, immunoglobulin A nephropathy; PCA, principal component analysis; N/C, no significance.

were most abundant in cells, indicating that mature miRNAs were the most common class of small RNAs in tissues. The miRNA sequences were mapped to databases containing human miRNAs and the quality of the transcriptome data is presented in Fig. 1D-H. Fig. 1D displays the alignment percentage for each sample. Sample alignment to hg38 was in a good range i.e. 75-92%. The average base quality score indicated that all reads in each sample had an average quality scoring from the 5'-3' end and distribution of how many reads have a particular base quality score on average (Fig. 1E-H). Fig. 1I and J indicate the quantification of the coverage breakdown of miRNAs; the percentage of fully within miRNAs in healthy participants was 5.61% of total RNAs and that in patients with IgAN was 4.35% of total RNAs in this study.

Significant DEMs in tissue samples of patients with IgAN. Gene expression signatures occur due to cellular perturbations, such as drug treatments, gene knockdown or diseases, and may be quantified using DGE methods comparing the gene expression between two groups of samples to identify significantly expressed genes altered in the perturbation. To identify miRNAs associated with the development of IgAN, miRNAs that were significantly differentially expressed were assessed (P<0.05 and FC ≥±2). Furthermore, to identify the similarity of biological samples in RNA-seq datasets, gene expression values were transformed into principal components (PCs), a set of linearly uncorrelated features representing the most relevant sources of variance in the data. The PCs scatter plot of the data for given samples is presented in Fig. 2A. Each point represents each sample and samples with similar gene expression profiles came closer in the three-dimensional space. Fig. 2B presents the hierarchical clustering data of differentially expressed genes in the analysis. Differential expression analysis of the set revealed that a total of 67 miRNAs were identified. Of the 67 miRNAs, 25 were significantly dysregulated: 10 were upregulated and 15 miRNAs were downregulated with a significant

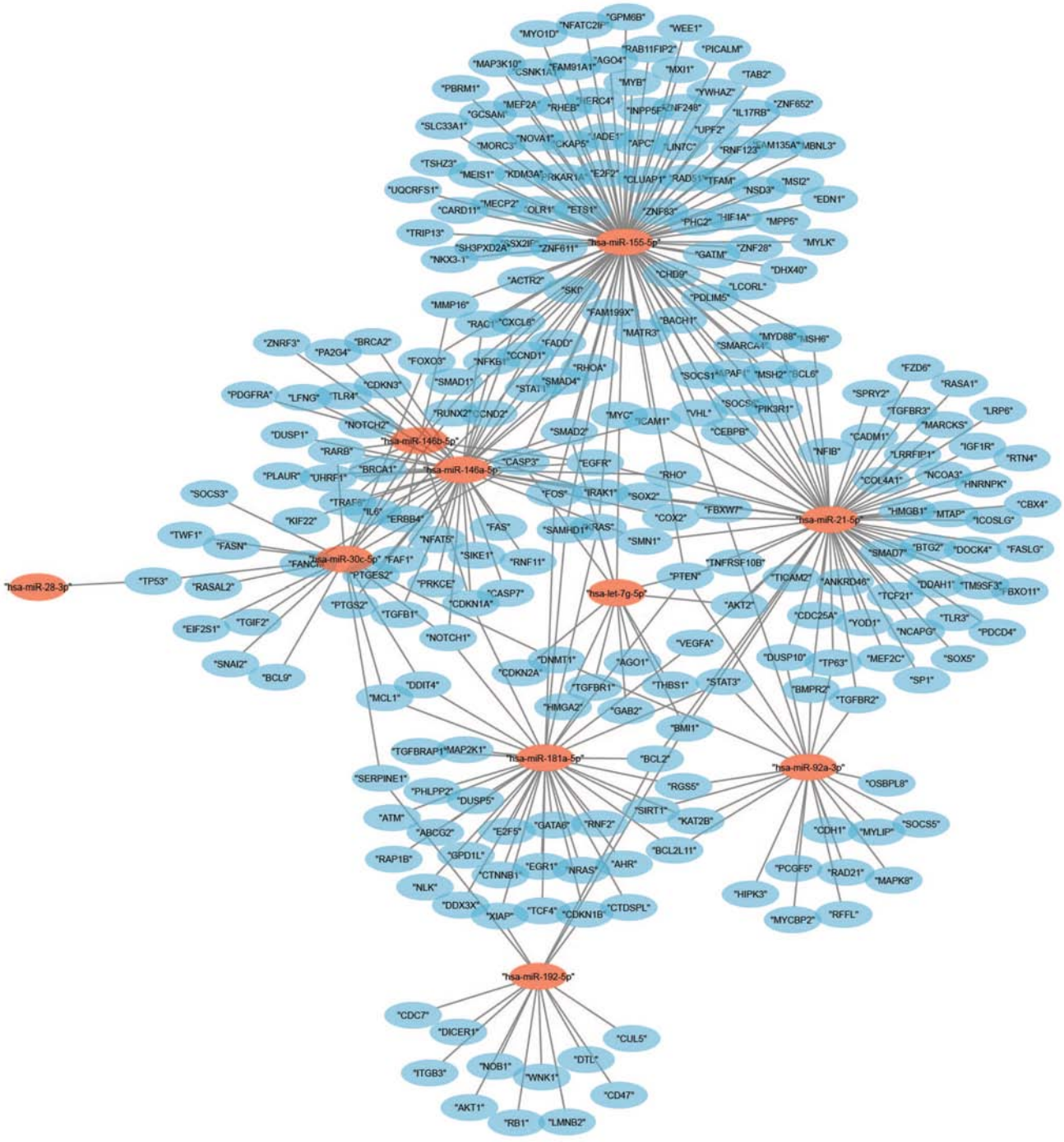

Figure 3. Regulatory network of upregulated miRNAs and their target genes. Red circles indicate the miRNAs, blue circles represent target genes and the lines indicate the interaction between miRNA and their target genes. miRNA/miR, microRNA.

P-value of ≤0.05 and Log2FC ≥±2 in tissues from patients with IgAN as compared with healthy participants (Fig. 2C). A list of significantly dysregulated miRNAs, comprising upregulated and downregulated miRNAs, with the FC and P-values, is provided in Table II. A pie chart indicating distributions of significantly differentially expressed up- and downregulated miRNAs in patients with IgAN as compared with healthy participants is displayed in Fig. 2D.

Functional prediction for upregulated genes. The top 10 upregulated miRNAs were first compiled in order to clarify the probable functions of DEMs in IgAN. With regard to these, hsa-miR-21-5p was significantly upregulated in patients with IgAN, whereas hsa-miR-146b-5p, hsa-miR-155-5p and hsa-let-7g-5p displayed substantial abundance in both healthy and test samples (IgAN) (Table IIB). When miRNAs target the 3'-UTRs of mRNAs, the stability of the mRNAs is decreased or translation efficiency is impeded. A total of 990 potential target genes were identified from the top 10 upregulated miRNAs and furthermore, an interaction network for miRNAs-target genes was created (Fig. 3). The largest subnetwork was found for miR-155-5p, which included 375 potential target genes.

The gene functions were examined by performing KEGG analysis and GO enrichment in terms of BP, CC and MF, as

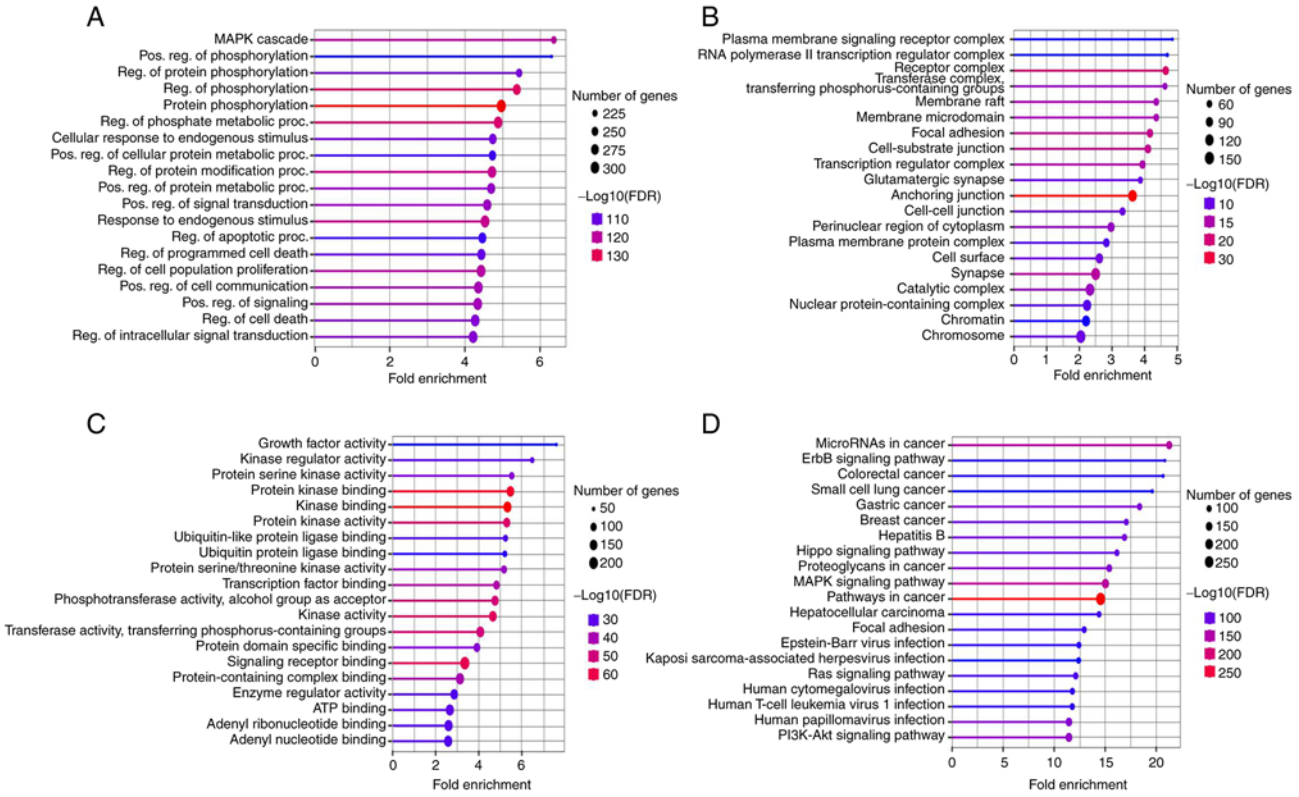

Figure 4. Illustration of Gene Ontology enrichment and KEGG pathway analysis for the top 10 upregulated miRNAs. Gene Ontology enrichment analysis in the categories of (A) Biological Process, (B) Cellular Component and (C) Molecular Function. (D) Top 20 enriched KEGG pathways of potential target genes for the upregulated miRNAs. The size of the points in the graph indicates the number of differential genes enriched and the color indicates the FDR value. FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA.

well as their associated pathways. The FDR cut-off was set at 0.05 and the top 20 significantly enriched terms were displayed for BP, CC and MF (Fig. 4). Most of the genes in the category BP were active and related to responses to the MAPK pathway, positive regulation of phosphorylation, protein phosphorylation and regulation (Fig. 4A). In the category CC, the majority of the genes were associated with 'Plasma membrane signaling receptor complex' and 'RNA polymerase II transcription regulator complex and receptor complex' (Fig. 4B). The terms with the maximum number of genes involved were 'Growth factor activity', 'Kinase regulator activity' and 'Protein serine kinase activity' in the MF category (Fig. 4C). Furthermore, KEEG pathways analysis revealed that the upregulated miRNA target genes were predominantly involved in 'Cancer pathways', 'ErbB signaling cascade' and in 'Colorectal cancer pathways' (Fig. 4D).

Functional prediction for downregulated genes. The top 15 miRNAs that were downregulated are presented in Table IIC; miR-127-3p had the highest abundance and miR-486-5p had the most significant downregulation in patients with IgAN. The 15 downregulated miRNAs have >1,600 target genes and the connections of these genes were presented in a network including the interactions between them (Fig. 5). The regulatory network revealed that hsa-miR-29c-3p had the largest subnetwork, which contained 245 potential target genes, while hsa-miR-1307-3p did not have any regulatory network.

The gene functions for the downregulated miRNAs were examined by performing KEGG analysis and GO enrichment in the categories BP, CC and MF, as well as their associated pathways. Most of the genes in the BP group were active and related to responses to the transmembrane receptor 'Protein tyrosine kinase signaling pathway' and 'Positive regulation of phosphorylation and phosphorus metabolic process' (Fig. 6A). However, in the category CC, most of the genes were involved in 'Plasma membrane signaling receptor complex', 'Glutamatergic synapse' and 'Membrane raft' (Fig. 6B). In the category MF, the genes mostly covered 'Transmembrane receptor protein kinase activity', 'Tyrosine kinase' and 'Growth factor binding' functions (Fig. 6C). Furthermore, KEGG pathway analysis revealed that 'Proteoglycans in cancer', 'miRNA in cancer' and 'Hippo signaling pathway' are the key pathways associated with the majority of genes (Fig. 6D).

Discussion

IgAN is the most commonly occurring type of glomerulonephritis worldwide in adults, with an estimated frequency of 2.5 per 100,000 individuals per year (25). Although IgAN is characterized by the aberrant glycosylation and deposition of IgA in mesangial areas, the exact pathogenesis remains elusive. Renal biopsy is the standard and most accurate method for IgAN diagnosis and disease assessment. However, it is difficult to perform due to haematuria and invasiveness. It has been indicated that miRNAs provide the possibility of non-invasive diagnosis of IgAN, as they have important roles in the occurrence and development of IgAN (25). miRNAs are also of certain value in predicting the disease prognosis. In 2010, Wang *et al* (26) described the relation of urinary sediment miR-192 in IgAN

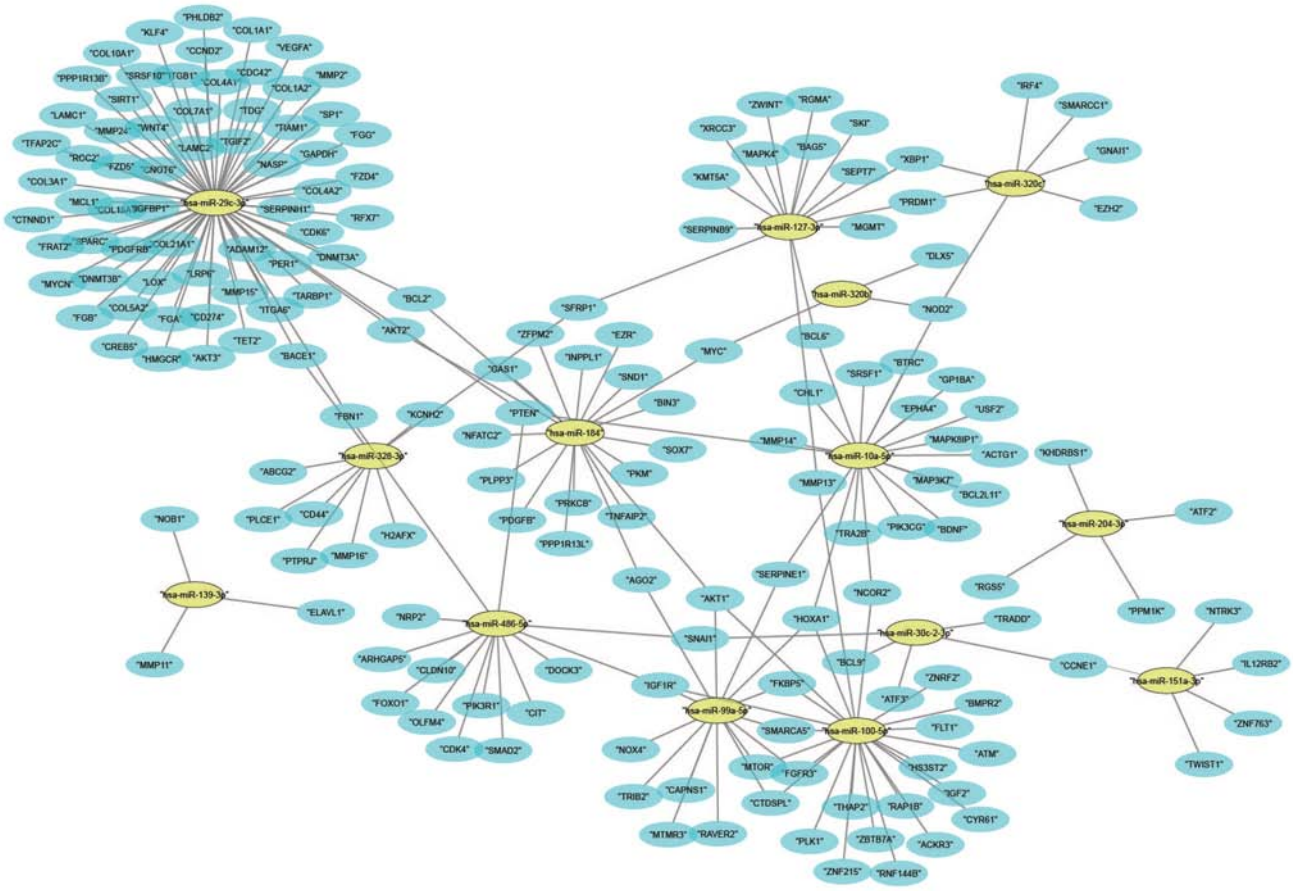

Figure 5. Regulatory network of downregulated miRNAs and their target genes. Yellow circles represent the miRNAs, blue circles represent the downregulated target genes and the lines indicate the interaction between miRNAs and their target genes. miRNA/miR, microRNA.

with the severity of renal injury and IgAN prognosis, which provided novel ideas for the study of miRNAs in IgAN. The deregulation of miR-148b was a key factor in the pathogenesis of IgAN, which may result in galactose-deficient IgA1 in IgAN, as reported by Serino *et al* (27) in 2012. Since then, studies of miRNAs have mainly focused on the mechanisms by which miRNAs influence the pathogenesis of IgAN, the association between miRNAs and the severity of IgAN and the relationship between miRNAs and the prognosis of IgAN (27).

miRNAs have a vital role in regulating various cellular processes, such as cell migration, proliferation and apoptosis. miRNAs are stable, ubiquitous and abundant in body fluids and cells; hence, miRNAs may be considered good biomarkers as well as drug targets (28,29). According to previous studies, miR-148b, let7b, miR-146a, miR-155, miR-21, miR-200 and miR-429 miRNAs may be involved in IgAN development (30). At the time of writing, 2,885 published articles were identified using the search terms 'kidney' and 'microRNA' in PubMed [US National Library of Medicine (NLM) at the National Center for Biotechnology Information (NCBI); https://pubmed.ncbi.nlm.nih.gov] in the last 5 years, but the exact role of miRNA expression in the pathogenesis of IgAN has not been well explored. In the present study, small RNA-seq was performed for the identification of additional functional miRNAs and the results demonstrated that nearly 75% of miRNAs were present in renal tissues from both healthy individuals and patients with IgAN. Among the 25 significant DEMs, miR-21-5p (31,32) and miR-155-5p (22,28) were previously reported to be associated with IgAN. However, the other three miRNAs, miR-146a, miR-192 and miR-146b, were newly identified in patients with IgAN. The immune regulator miR-146a is known to be upregulated in patients with IgAN (22,28) and is significantly related to inflammatory cell infiltration and interstitial lesions. In addition, miR-192 is also associated with interstitial fibrosis and epithelial-to-mesenchymal transition (28,33). In the present study, amongst the top 10 upregulated miRNAs, miR-21-5p had the maximum FC, followed by miR-146b-5p and miR-155-5p. miR-181a-5p exhibited the most abundant expression in patients with IgAN as well as in healthy individuals, followed by miR-30c-5p. Amongst the top downregulated miRNAs, miR-486-5p had the maximum FC, followed by miR-127-3p and miR-320c, and miR-184 exhibited the most abundant expression followed by miR-100-5b and miR-320c in both healthy individuals as well as patients with IgAN. The important factor for the progression of IgAN is inflammation). miR-146a was previously reported to inhibit inflammation by targeting tumor necrosis factor receptor-associated factor 6 (TRAF6), TLR4 and IL-1 receptor-associated kinase 1 and suppressed NF-κB signaling (34). In addition, the expression of miR-146a is also inhibited in TGF-β1-Smad signaling and TRAF6-NF-κB signaling pathways (35,36). In the present study, the expression of miR-146a-5p and miR-146b-5p was also identified in renal tissues of patients with IgAN and the upregulation of these two miRNAs was also found in tissue samples targeting most of the

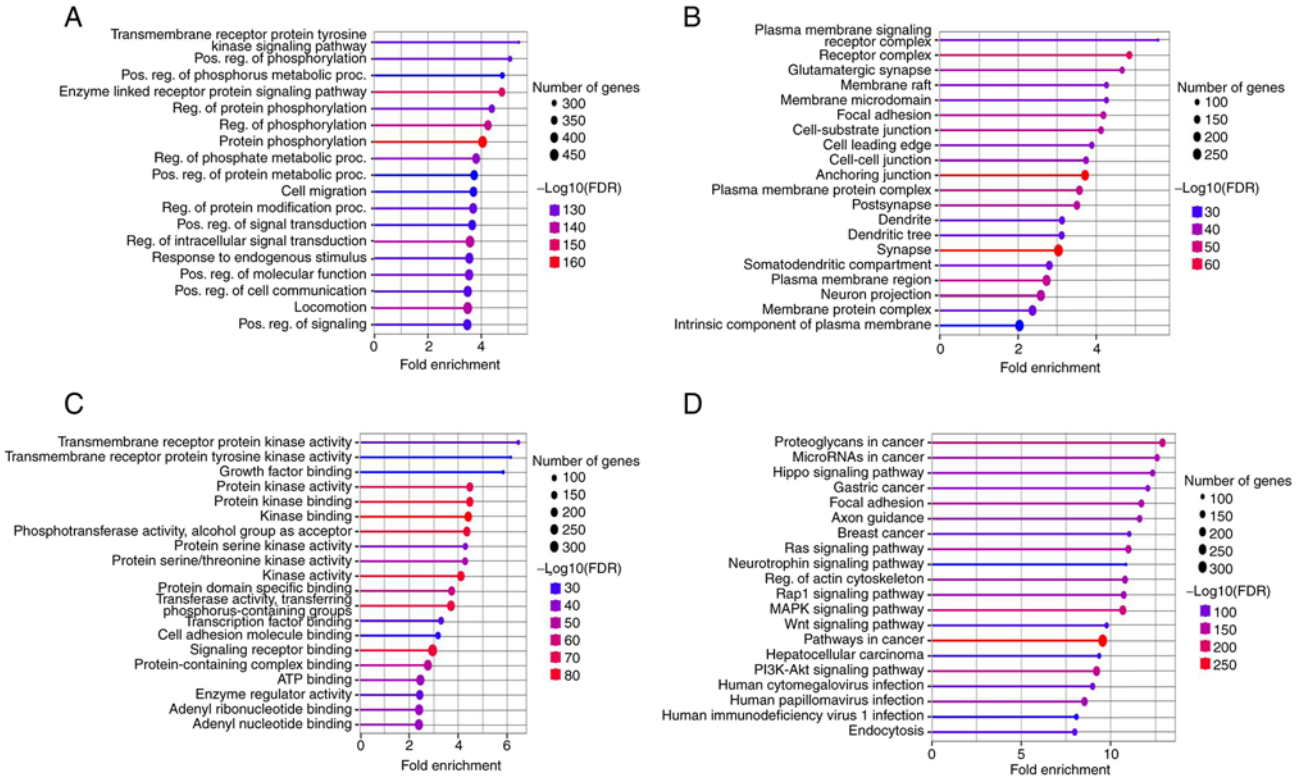

Figure 6. Illustration of Gene Ontology enrichment and KEGG pathway analysis for the top 15 downregulated miRNAs. Gene Ontology terms in the categories of (A) Biological Process, (B) Cellular Component and (C) Molecular Function. (D) Top 20 enriched KEGG pathways of potential target genes for the downregulated miRNAs. The size of the points in the graph indicates the number of differential genes enriched and the color indicates the FDR value. miRNA, microRNA; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

genes along with TRL4, TRAF6, TGF-β1, SMAD and NF-κB. miR-146a and miR-146b may decrease renal fibrosis by inhibiting profibrotic and inflammatory signaling pathways and may be used as another therapeutic target for renal fibrosis treatment; these findings are in line with the previously published work by Ichii *et al* (34) and Lin *et al* (35). The present study reported, for the first time, that the 5 upregulated miRNAs, miR-181a-5p, miR-28-3p, let-7g-5p, miR-30c-5p, miR-92a-3p are interconnected with IgAN disease. Numerous studies have indicated that the overexpressed miRNAs may deregulate phosphatase and tensin homolog (PTEN) and silencing PTEN at the post-transcriptional level, considering another form of epigenetic modification (37). Zhang *et al* (38) reported that miR-21 directly bound to the 3'-UTR of PTEN and promoted the proliferation, migration and invasion of gastric cancerous cells. Furthermore, the expression of PTEN is increased in the PI3K/AKT/mTOR signaling pathway by the antisense oligoneucleotides against PTEN, leading to reverse effects on the cellular microenvironment of gastric cancerous cells (38). In the present study, PTEN was found to be the target gene of miR-21-5p, miR-155-5p, miR-92a-3p and let-7g-5p miRNAs in the tissue samples of patients with IgAN; however, the association of let-7g-5p and PTEN in patients with IgAN remains to be further investigated. The dysregulation of miR-92a-3p is known to have a role in tumorigenic processes; therefore, it is associated with tumor progression and prognosis. In 2018, Zhu *et al* (39) demonstrated that miR-92a-3p blocks the progression of Wilms' tumor by targeting notch receptor 1 (NOTCH1). In the present study, NOTCH1 was also found as a targeted gene of miR-92a-3p, suggesting NOTCH1 is the main target of miR-92a-3p for the inhibition of the proliferation, migration and invasion of tumor cells.

KEGG pathway and gene enrichment analysis identified that the MAPK pathway, Cancer pathways, the ErbB signaling cascade, Colorectal cancer pathway, Protein tyrosine kinase signaling pathway, miRNAs in cancer, Proteoglycans in cancer and Hippo signaling pathways were the most significantly enriched key pathways associated with the majority of genes (Figs. 4D and 6D) and also involved in regulating cell proliferation or IgAN development. These findings suggest that miRNAs that are differentially expressed may be involved in the progression and deposition of IgAN through their target genes directly or by regulating various signaling pathways. Studies have indicated that renal ischemia/reperfusion injury (I/R) frequently occurs in kidney transplantations and acute kidney injuries and the upregulation of miR-30c-5p has a reno-protective effect against renal I/R by reducing inflammation (40). Schneider *et al* (41) reported that miR-28-3p is known to control the proliferation of cells and is downregulated in B-cell lymphomas and acts as a tumor suppressor. It is already reported that tumor suppressor p53 has a major role in preventing tumors and the activity or function of p53 may be regulated by miRNAs through the direct suppression of p53 or its regulators in cells (42). In the present study, p53 was found to be the only target of miR-28-3p, suggesting miR-28-3p may be involved in the prevention of tumor progression in patients with IgAN. Wu *et al* (43) also demonstrated that the downregulation of miR-127-3p has an inhibitory effect

on IFN-1 signalling and may also inhibit the induction of interferon-stimulated response element and gamma-activated sequence-mediated gene expression in the renal tissues of patients with lupus nephritis. In the present study, miR-127-3p expression was significantly downregulated, which is in agreement with the previously published reports. On the other hand, miR-127-3p has been indicated to target BCL6, a key transcriptional factor for the differentiation of follicular T-helper cells, and is involved in immune disorders of various autoimmune diseases (44-46). This further supports that miR-127-3p may serve as a therapeutic target for autoimmune diseases. However, to identify the unique biomarkers for IgAN, further research is required to compare the variations in the expression of these miRNAs with other types of kidney disorders.

Over the last few years, the potential value of miRNAs as biomarkers in IgAN has increasingly developed. Identification of aberrant signaling pathways in IgAN may help to uncover the fundamental molecular mechanisms behind those signaling pathways linked to IgAN and to identify more promising molecular candidates with effective diagnostic and prognostic value. These findings further elucidate the pathogenesis of IgAN and help to develop personalized treatments for IgAN. Reports have indicated that circulating miRNA-mediated gene regulation is dynamic and exhibits a bio-fluids-specific profile in relation to a pathophysiological state. Besides, the attractiveness of circulating miRNAs as biomarkers is associated with the tissue-specific nature of miRNA expression. This gives rise to a specific mechanism for intercellular communication and an application of miRNAs as diagnostic/prognostic biomarkers (47,48). Integrated bioinformatics analysis revealed that the pathways identified in the present study may have important roles in the pathogenesis of IgAN. The present study is a pilot study to determine whether miRNAs are viable therapeutic targets for IgAN in the Indian population, and the findings will serve as a springboard for data collection in a larger sample. However, a restriction is the limited sample size. Therefore, additional research using larger sample sizes is necessary to confirm the biomarkers and look into the molecular mechanisms underlying the aberrant signalling pathways that led to the development of IgAN.

In conclusion, the current investigation discovered 25 miRNAs that were differentially expressed and linked to IgAN. Many of these connections are novel and may have a significant role in the pathogenesis of IgAN. In addition, the DEMs miR-181a-5p, miR-92a-3p, let-7g-5p, miR-28-3p and miR-30c-5p may govern the development of IgAN by controlling the behaviour of tissues or IgA deposition via targeting the signaling pathways. It is of great interest to the area of nephrology that these miRNA panels or individual miRNAs are employed as circulating miRNA biomarkers or in conjunction with other biomarkers and therapeutic targets for IgAN diagnosis and therapy to increase the diagnostic value.

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Availability of data and materials

The datasets generated, used and/or analyzed during the present study are available from the corresponding author upon reasonable request. The NGS data are available from the Mendeley Data repository [http://dx.doi.org/10.17632/j7bz9v33w8.1].

Authors' contributions

AT designed the study, performed all the experiments and data analysis and prepared the manuscript. PCY and RVV performed the bioinformatics analysis, participated in its interpretation and revised the manuscript. AS performed the histopathological analysis. SKR supervised and was involved in the clinical diagnosis, surgical resection and providing the patients' samples. AT and PCY verified the authenticity of the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research study was approved by the Institutional Research and Ethics Committee of AIG Hospital (Hyderabad, India; AHF/AIGH-IRB: 02/47/2021) and written informed consent was obtained from all patients included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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