Circulating MicroRNAs as Potential Biomarkers for Nephrotic Syndrome

Weiguo Sui,[1] Hua Lin,[1] Haiyan Li,[1] Qiang Yan,[1] Jiejing Chen,[1] Yong Dai[2]

[1]Department of Nephrology, 181st Hospital, Guangxi Key Laboratory of Metabolic Diseases Research, Guilin, China
[2]Shenzhen People's Hospital, The Second Clinical Medical College of Jinan University, Shenzhen, China

Keywords. blood circulation, biological markers, microRNAs, nephrotic syndrome, real-time polymerase chain reaction

Introduction. Recent studies have shown that serum microRNAs have specific expression patterns in some diseases, indicating the potential of using microRNAs to aid diagnosis. This study estimated the levels of microRNAs in patients with nephrotic syndrome compared with healthy controls.
Materials and Methods. In this study, real-time quantitative polymerase chain reaction was used to explore whether there were different expression levels of miR-181a, miR-483-5p, and miR-557 in the serum of patients with nephrotic syndrome subtypes and healthy controls. We measured the three microRNAs in 40 patients with nephrotic syndrome and 16 healthy controls.
Results. The circulating levels of miR-483-5p and miR-557 were not significantly upregulated or downregulated, whereas miR-181a was significantly upregulated in patients with nephrotic syndrome as compared with healthy controls.
Conclusions. We found that circulating miR-181a had a significantly different expression and could be an effective means to aid diagnosis of nephrotic syndrome. This microRNA is an attractive candidate as a biomarker for nephrotic syndrome.

IJKD 2014;8:371-6
www.ijkd.org

Original Paper

INTRODUCTION

Nephrotic syndrome (NS) is not a single disease but a syndrome, a set of symptoms and signs that tend to occur together. Nephrotic syndrome has 4 common subtypes: mesangial proliferative glomerulonephritis, minimal change NS, focal segmental glomerulosclerosis, and membranous nephropathy. Nephrotic syndrome can be caused by one or more different diseases that increase the permeability of the glomerular basement membrane and induce massive plasma protein loss. Nephrotic syndrome is characterized by massive proteinuria, which leads to hypoproteinemia, hyperlipidemia, and edema.[1] In addition, NS has many complications, including infection, thrombosis, acute kidney failure, renal tubule hypofunction, abnormal calcium metabolism, and endocrine disturbance.

It is important to develop early detection methods for the etiopathogenesis of NS. The ideal biomarker should be easily accessible, so that it can be sampled noninvasively, and sensitive enough to detect the early presence of NS. To date, the exact mechanisms involved in NS are not completely understood. To search for these biomarkers, such as interleukin-2 and mutations of the *NPHS2* gene have been attempted.[2,3] For example, serum soluble interleukin-2 receptor and multidrug resistant gene-1 gene expression levels are early predictors of steroid resistance in NS for early control of disease by immediate introduction of cytotoxic drugs.[4] Mutations of *NPHS2* gene are frequent among Iranian children with steroid-resistant nephrotic syndrome.[5] Lots of work has been done in searching for biomarkers, from the aspects of DNA and protein, expecting

to illustrate the mechanism of NS and find ideal biomarkers for diagnosis and precaution of NS.

Currently, serum is one of the best subjects for research because of its easy collection and informative record of physiological condition.[6,7] MicroRNAs have become a hot topic of research since the first 2 microRNAs were discovered.[8] MicroRNAs are small, noncoding RNAs with 18 to 24 nucleotides that regulate gene expression and play important roles in a variety of cellular functions.[9,10] MicroRNAs can regulate gene expression at the posttranscriptional level by blocking translation or degradation of messenger RNA.[11,12] Recent studies have suggested that microRNAs play very important roles in development, apoptosis, and metabolism.[13-15] Recent blood-based microRNA profiling studies have reported their presence in serum and plasma. This finding generated the hypothesis that circulating microRNAs may potentially be used as noninvasive biomarkers for diagnosis of cancer and other diseases.[16-20] MicroRNAs have many ideal characteristics as biomarkers, including their inherent stability and resilience; for example, they are known to be well preserved in tissue samples even after several years and can be efficiently extracted from and quantified in specimens.[21]

Some studies have suggested that microRNAs are released to the blood in microvesicles or apoptotic bodies.[22,23] A recent study showed that microRNAs are released by a ceramide-dependent secretory machinery and that these secretory microRNAs are transferable and functional in the recipient cells.[24] However, the secretory mechanisms and the biological function of circulating microRNAs need further investigation.

In this study, our aim was to estimate the levels of microRNAs in patients with NS compared with healthy controls. We determined the circulating levels of microRNAs in patients with NS by performing real-time quantitative polymerase chain reaction (PCR). The primary aim was to investigate whether renal-specific microRNAs were detectable and altered in the circulation of NS patients compared with healthy controls.

METHODS AND METHODS

Samples

Serum samples were taken from 40 patients with NS at a public hospital. They had been diagnosed with mesangial proliferative glomerulonephritis, minimal change NS, membranous nephropathy, or focal segmental glomerulosclerosis. The diagnoses were confirmed using clinical evidence. Patients whose diagnosis could not be confirmed were excluded. In addition, 16 samples from healthy people were taken at the physical center of the hospital. These diagnoses were also confirmed using clinical evidence. We divided these samples into 5 groups based on the above diagnoses.

All samples were collected after receiving informed consent from the participants. All study protocols abided by the Helsinki Declaration on ethical principles for medical research involving human subjects.

Sample collection and storage

Whole blood from the participants was collected into tubes. All blood was processed for serum by centrifugation within 3 hours. The serum was then stored at -80°C.

RNA Isolation

Total RNA was extracted from serum using Trizol (Invitrogen). The concentration and quality of total RNA was measured using the ratio of the ultraviolet absorbance at 260 nm and 280 nm (A260/280). If this ratio was approximately 2.0, a sample was considered acceptable. A ratio less than 1.8 indicates protein or other contamination. In this study, all ratios were higher than 1.8, indicating that the RNA extraction was successful. All RNAs within a sample group were pooled and used for microRNA isolation and real-time quantitative PCR. Isolation of microRNA from the total RNA was performed using SYBR Green PCR Master Mix (Toyobo, Japan) according to the manufacturer's instructions.

Candidate MicroRNA Selection

The potential renal-related microRNAs were chosen according to a previous microRNA microarray study.[25] In that study, Sui and coworkers demonstrated that microRNAs were significantly differently expressed in patients rejecting kidney transplantation compared with healthy controls. For example, miR-181a, miR-483-5p, and miR-557 were differentially expressed in the microRNA microarray. These were judged to be potential biomarkers for diagnosis and probable factors

involved in the pathogenesis of rejection. It has been reported that miR-181a is associated with lupus nephritis in renal biopsy samples, meaning it is linked to kidney damage.[26] MiR-181a was overexpressed in human embryonic kidney cells.[27] Kidney diseases are a family of disorders with heterogeneous etiologies. Therefore, we hypothesized that these three microRNAs might have an influence on kidney diseases.

Candidate MicroRNA Quantification

Real-time quantitative PCR analysis (ABI PRISM® 7500 Sequence Detection System, Life Technologies, USA) was used to quantify miR-181a, miR-483-5p, and miR-557 expression according to the manufacturer's protocol. Cycle threshold (Ct) is the cycle number at which the fluorescence signal passed the appropriate threshold. The cycle parameters for the PCR reaction were 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 microRNA to U6 RNA was calculated using the equation $2^{-\Delta Ct}$, where ΔCt = Ct microRNA − Ct U6. RNA was reverse transcribed to complementary DNA with gene-specific primers. Real-time quantitative PCR primers are listed in Table 1. The microRNAs analyzed included miR-181a, miR-483-5p, and miR-557.

Statistical Analyses

The resulting microRNA expression levels were log transformed for analysis. Quantitative data were expressed as the mean ± standard deviation. Expression levels of microRNAs were analyzed using the $2^{--\Delta\Delta Ct}$ method. Quantitative data were compared using the *t* test among groups, and the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, Ill, USA) was used for analysis of variance between the 5 groups. Results with a *P* value less than .05 were considered significant.

RESULTS

Detailed clinical and demographic information is listed in Table 2. To explore circulating microRNAs as novel biomarkers for NS, we measured the levels of three target microRNAs (miR-181a, miR-483-5p, and miR-557; Table 3) in the circulation of 40 NS patients compared with 16 normal subjects. The expression of one renal-associated microRNA (hsa-miR-181a) was very different between patients and controls, corresponding to an average fold-change of 19.1722 (minimal change NS), 20.0893 (mesangial proliferative glomerulonephritis), 20.201 (membranous nephropathy), and 32.9247 (focal segmental glomerulosclerosis), respectively ($P < .05$; Tables 3 and 4). In contrast, expression of circulating miR-483-5p and miR-557 did not differ significantly between NS patients and

Table 1. Reverse-transcription and Real-time Quantitative Polymerase Chain Reaction Primers

Gene Name	Accession Number	Primer Sequence	Length (Base Pairs)	Annealing Temperature, °C
miR-181a	MIMAT0000256	5'ACACTCCAGCTGGGA ACATTCAACGCTGTCGG3'	73	60
miR-483-5p	MIMAT0004761	5' ACACTCCAGCTGGGA AGACGGGAGGAAAGAAG3'	72	60
miR-557	MIMAT0003221	5'ACACTCCAGCTGGGGTTT3'	73	60
U6		5'GCACGGGTGGGCCTT3'	94	60

Table 2. Clinical Characteristic of Patients With Nephrotic Syndrome and Healthy Controls

Characteristic	Group				
	Normal Control (n = 16)	Minimal Change Nephrotic Syndrome (n = 10)	Mesangial Proliferative Glomerulonephritis (n = 10)	Membranous Nephropathy (n = 10)	Focal Segmental Glomerulosclerosis (n = 10)
Age, y	40.23 ± 2.31	19.97 ± 2.10	32.78 ± 8.64	25.42 ± 10.64	41.33 ± 14.96
Serum total cholesterol, μmol/L	3.26 ± 1.80	13.16 ± 3.52	8.07 ± 3.94	8.50 ± 2.74	11.16 ± 5.16
Triglyceride, mmol/L	1.25 ± 0.81	2.74 ± 0.93	2.50 ± 0.61	3.39 ± 1.96	4.72 ± 3.16
Total protein, μmol/L	68.07 ± 7.61	38.15 ± 5.81	41.99 ± 9.27	31.72 ± 3.98	47.71 ± 1.09
24-hour urine protein, g/d	0.06 ± 0.01	4.11 ± 3.61	3.27 ± 2.33	3.98 ± 3.22	3.82 ± 3.08

Table 3. Serum MicroRNA Expression in Nephritic Syndrome and Normal Control Groups*

MicroRNA	Group					Ratio								
	Normal Control	MCNS	MsPGN	MN	FSGS	MCNS/NC	MsPGN/NC	MN/NC	FSGS/NC	MsPGN/MCNS	MN/MCNS	FSGS/MCNS	MN/MsPGN	FSGS/MsPGN	FSGS/MN
miR-181a	7.9313	19.1722	20.0893	20.201	32.9247	2.4173	2.5329	2.5469	4.1512	1.0478	1.0536	1.7173	1.0055	1.6389	1.6298
miR-483-5p	7.3519	9.6046	8.0297	6.8097	7.0477	1.3064	1.0921	0.9262	0.9586	0.8360	0.7090	0.7337	0.8480	0.8777	1.0349
miR-557	6.4563	7.7235	7.6014	7.3827	6.3025	1.1962	1.1773	1.1434	0.9761	0.9841	0.9558	0.8160	0.9712	0.8291	0.8536

*MicoRNAs are arranged according to microRNA names. If the ratio is higher than 2 or less than 0.5, the difference is recognized as significant. If the ratio higher than 1, the difference is upregulation; otherwise, it is downregulation. MPGN indicates mesangial proliferative glomerulonephritis; MCNS, minimal change nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; and MN, membranous nephropathy.

controls (Table 3 and Table 4). We also compared the expression of microRNAs pairwise between subtypes of NS. These data are listed in Table 3 and Table 4.

DISCUSSION

As research into microRNAs continues, new microRNAs are being continually discovered, and their functions are being confirmed one by one. Inherent characteristics of microRNAs, such as their lower complexity, tissue-specific expression profiles, and stability, make these molecules ideal biomarkers to indicate various physiological and pathological states. The roles and functions of microRNAs may have broader implications; for example, microRNAs may serve as novel biomarkers for kidney disease prediction. Recent studies have suggested that microRNAs are potent regulators of the immune response, possessing great potential as both biomarkers and therapeutic targets,[28,29] and are remarkably stable in the bloodstream.[30] Circulating microRNAs have emerged recently as candidate biomarkers for disease, particularly cancer. In this study, serum samples were taken from 40 NS patients, demonstrating that microRNA can be detected in human serum. We divided these samples into groups and used real-time quantitative PCR to explore whether there were different expression levels of miR-181a, miR-483-5p, and miR-557 in NS and healthy controls. We found that these three microRNAs exhibited different expression patterns. MiR-483-5p and miR-557 were not significantly upregulated or downregulated, whereas miR-181a was significantly upregulated in NS patients versus healthy controls. The circulating levels of miR-181a, miR-483-5p, and miR-557 showed no significant upregulation or downregulation among subtypes of NS.

Our study indicates that microRNAs with NS-specific expression could be effective aids in the diagnosis of NS. This study was the first report of circulating microRNAs in NS patients, and our results demonstrate that renal-associated microRNAs in serum can potentially serve as novel biomarkers for NS. These three candidate circulating microRNAs are attractive candidates as biomarkers and therapeutic targets for NS, meriting further attention to this area. In particular, the circulating level of miR-181a was significantly upregulated in patients with NS. Therefore, circulating miR-181a

Table 4. Comparison of Relative Levels of Nephrotic Syndrome–associated Serum MicroRNAs in Normal Control and Nephrotic Syndrome Samples as Determined by Real-time Quantitative Polymerase Chain Reaction*

Target MicroRNA	Fold Change				
	Normal Control (n = 16)	Minimal Change Nephrotic Syndrome (n = 10)	Mesangial Proliferative Glomerulonephritis (n = 10)	Membranous Nephropathy (n = 10)	Focal Segmental Glomerulosclerosis (n = 10)
miR-181a	7.93 ± 4.91	19.17 ± 13.42†	20.09 ± 19.72†	20.20 ± 10.01†	32.92 ± 26.73†
miR-483-5p	7.35 ± 3.49	9.60 ± 7.86	8.03 ± 9.05	6.81 ± 3.24	7.05 ± 6.37
miR-557	6.46 ± 2.90	7.72 ± 6.40	7.60 ± 4.12	7.38 ± 8.54	6.30 ± 4.52

*Fold change was relative to controls and presented as the mean ± standard deviation.
†$P < .05$

might present a potential candidate biomarker to distinguish between NS and healthy controls. It must be remembered that we chose only three microRNAs and cannot exclude the possibility of other specific circulating microRNAs. The sensitivity and specificity of other potential microRNAs need to be further investigated, and much research is needed to confirm the results. To validate these findings and further explore the potential of circulating microRNAs as novel and clinically useful biomarkers for kidney diseases, further evaluation of blood-based microRNAs in NS and other kidney diseases is needed.

We searched for targets of the three selected microRNAs using TargetScan Release 4.2: April 2008 at www.targetscan.org. The predicted targets of each microRNA are listed in Table 5. Moreover, we can predict their targets with target predictions tools such as miRanda available at http://www.microrna.org and miRBase available at http://microrna.sanger.ac.uk/targets/v2/etc.[31]

CONCLUSIONS

Our study found that circulating miR-181a, with a significantly different expression in NS patients versus controls, could be an efficient means to aid the diagnosis of NS and is an attractive candidate as a biomarker for NS. This is a renal-specific microRNA that was detectable and altered in the circulation of NS patients. Our analysis suggests that changes in the circulating levels of microRNAs might influence critical pathways involved in NS development. This finding will help elucidate possible pathogenic mechanisms of NS. Our work may lead to novel methods to diagnose and treat NS and provide a novel research approach for other diseases.

ACKNOWLEDGEMENTS

We thank the patients with NS and healthy volunteers who participated in this study.

FINANCIAL SUPPORT

This study was financially supported by Natural Science Fund of Guangxi, People's Republic of China (Grant No, 2012GXNSFDA053017).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Hull RP, Goldsmith DJ. Nephrotic syndrome in adults. BMJ. 2008;336:1185-9.
2. Otukesh H, Ghazanfari B, Fereshtehnejad SM, et al. NPHS2 Mutations in Children With Steroid-Resistant Nephrotic Syndrome. Iran J Kidney Dis. 2009;3:99-102.
3. Fotouhi N, Ardalan M, Jabbarpour Bonyadi M, et al. R229Q Polymorphism of NPHS2 Gene in Patients With Late-Onset Steroid-Resistance Nephrotic Syndrome: a Preliminary Study. Iran J Kidney Dis. 2013;7:399-403.
4. Youssef DM, Elbehidy RM, Abdelhalim HS, Amr GE. Soluble Interleukine-2 Recptor and MDR1 Gene Expression Levels as Inflammatory Biomarkers for Prediction of Steroid Response in Children With Nephrotic Syndrome. Iran J Kidney Dis. 2011;5:154-61.
5. Basiratnia M, Yavarian M, Torabinezhad S, Erjaee A. NPHS2 Gene in Steroid-resistant Nephrotic Syndrome:

Table 5. Predicted MicroRNA Targets From TargetScan Release 4.2: April 2008

MicroRNA Names	Target Genes	Target Genes Location	Conserved Targets
miR-181a	Human diphosphoinositol pentakisphosphate kinase 2 *(PIP5K2)*	5p21.1	1194
miR-483-5p	Serine/Arginin e-rich splicing factor 4 *(SRSF4)*	1p35.3	9
miR-557	BATB and CNC homology 1, basicleucine zipper transcription factor 2 *(BACH2)*	…	374

Prevalence, Clinical Course, and Mutational Spectrum in South-West Iranian Children. Iran J Kidney Dis. 2013;7:357-62.

6. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics. 2002;1:845-67.
7. Liotta LA, Ferrari M, Petricoin E. Clinical proteomics: written in blood. Nature. 2003;425:905.
8. Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403:901-6.
9. Vaucheret H. Plant ARGONAUTES. Trends Plant Sci. 2008;13:350-8.
10. Grosshans H, Filipowicz W. Proteomics joins the search for microRNA targets. Cell. 2008;134:560-2.
11. Pan W, Zhu S, Yuan M, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. J Immunol. 2010;184:6773-81.
12. Muljo SA, Kanellopoulou C, Aravind L. MicroRNA targeting in mammalian genomes: genes and mechanisms. Wiley Interdiscip Rev Syst Biol Med. 2010;2:148-61.
13. Mallory AC, Bouche N. MicroRNA-directed regulation: to cleave or not to cleave. Trends Plant Sci. 2008;13:359-67.
14. Visone R, Croce CM. MicroRNAs and cancer. Am J Pathol. 2009;174:1131-8.
15. Callis TE, Chen JF, Wang DZ. MicroRNAs in skeletal and cardiac muscle development. DNA Cell Biol. 2007;26:219-25.
16. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA. 2008;105:10513-8.
17. Gilad S, Meiri E, Yogev Y, et al. Serum MicroRNAs are promising novel biomarkers. PLoS One. 2008;3:e3148.
18. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997-1006.
19. Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol. 2009;112:55-9.
20. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg. 2010;251:499-505.
21. Li J, Smyth P, Flavin R, et al. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. BMC Biotechnol. 2007;7:36.
22. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011;108:5003-8.
23. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 2010;101:2087-92.
24. Iguchi H, Kosaka N, Ochiya T. Secretory microRNAs as a versatile communication tool. Commun Integr Biol. 2010;3:478-81.
25. Sui W, Dai Y, Huang Y, Lan H, Yan Q, Huang H. Microarray analysis of MicroRNA expression in acute rejection after renal transplantation. Transpl Immunol. 2008;19:81-5.
26. Te JL, Dozmorov IM, Guthridge JM, et al. Identification of unique MicroRNA signature associated with lupus nephritis. PLoS One. 2010;5:e10344.
27. Khanna A, Muthusamy S, Liang R, Sarojini H, Wang E. Gain of survival signaling by down-regulation of three key microRNAs in brain of calorie-restricted mice. Aging (Albany NY). 2011;3:223-36.
28. Li J, Wan Y, Guo Q, et al. Altered microRNA expression profile with miR-146a upregulation in CD4+ T cells from patients with rheumatoid arthritis. Arthritis Res Ther. 2010;12:R81.
29. Liu Z, Sall A, Yang D. MicroRNA: An emerging therapeutic target and intervention tool. Int J Mol Sci. 2008;9:978-99.
30. Ferracin M, Veronese A, Negrini M. Micromarkers: microRNAs in cancer diagnosis and prognosis. Expert Rev Mol Diagn. 2010;10:297-308.
31. Rajewsky N. microRNA target predictions in animals. Nat Genet. 2006;38:S8-13.

Correspondence to:
Yong Dai, PhD
Shenzhen People's Hospital, The Second Clinical Medical College of Jinan University, Shenzhen, 518020, China
Tel: +86 755 2562 6750
Fax: +86 755 2562 6750
E-mail: daiyong2222@gmail.com

Weiguo Sui and Hua Lin contributed equally to this work as first authors.
Received October 2013
Revised February 2014
Accepted March 2014