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The Association Between Plasma MicroRNA-451 Expression Levels and Chronic Kidney Disease in Children with β-Thalassemia Major

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Original Paper

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Introduction. Patients with β -thalassemia major (β -TM) had a high rate of glomerular dysfunction due to chronic anemia, iron overload, and chelation therapy. There is also evidence of proximal tubular damage, as almost all patients have various amounts of proteinuria. MicroRNAs are non-coding RNA molecules that regulate gene expression. In diabetes, a relative increase in renal microRNA-451 appeared to protect against diabetic kidney injury. This study aimed to investigate the association between miRNA-451 and the development of chronic kidney disease (CKD) in children with β-TM.

Methods. This study included 60 pediatric patients with β-TM and 30 healthy children as controls. We categorized patients into two groups according to the presence of CKD. Complete blood and reticulocyte counts, serum levels of ferritin, creatinine and glucose, and urine albumin/creatinine ratio (ACR) were measured. Plasma miRNA-451 expression level was measured by real-time quantitative reversed transcription PCR in all included children.

Results. miRNA-451 levels were significantly higher in β-TM (25.326 ± 12.191) as compared with controls (9.453 ± 5.753) ($P < .001$). Patients with β-TM and CKD had significantly lower miRNA-451 levels (19.72 ± 13.023) than those without CKD (30.933 ± 8.23). MiRNA-451 levels had significantly positive correlated with eGFR (r = 0.385 $P < .05$) and reticulocyte counts (r = 0.27, $P < .05$). Linear logistic regression analysis showed that low plasma microRNA-451 was a significant independent predictor of CKD.

Conclusion. miRNA-451 has a protective role against CKD development, and low plasma expression levels are associated with CKD in children with β-TM

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INTRODUCTION

Thalassemia is a group of inherited hemoglobin synthesis disorders that manifests as chronic hemolytic anemia and iron overload. β-Thalassemia is the most common monogenic disorder globally, with clinical manifestations, ranging from asymptomatic carriers to lifelong transfusion-dependent anemia.[1,2] Improvements in patient care and management have resulted in a significant increase in longevity of thalassemia patients and

a greater awareness of associated complications, such as kidney dysfunction.[3] Mechanisms of kidney injury in thalassemia patients include increased oxidative stress and lipid peroxidations due to chronic anemia and hypoxemia with subsequent renal tubular cell dysfunction.[4] Besides, iron overload leads to glomerular dysfunction adding to kidney injury.[2,4] Though glomerular filtration rate (GFR) reduction rarely occurs in children with β thalassemia major (β-TM), gradual deterioration of kidney function and progressive kidney damage may develop with age.[5] Several studies have investigated the role of renal biomarkers in early prediction of kidney dysfunctions in pediatric patients with β-TM during the last decades.[6-8]

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression.[9] Several human diseases are attributed to abnormal expression of miRNAs.[10] Additionally, the secretion of miRNAs into the extracellular fluids makes them potential biomarkers for various diseases.[11,12] Several miRNAs have been linked to erythropoiesis regulation.[13] The role of miRNA-451 in patients with β-TM was previously investigated, and it was discovered to be expressed in patients with hemolysis, with its level correlated to disease severity.[14] Few previous studies had reported that miRNA-451 has a protective role against the development of diabetic nephropathy.[15] This study was conducted to investigate the association between plasma miRNA-451 expression levels and chronic kidney disease (CKD) in children with β-TM.

MATERIALS AND METHODS

This study was carried out between October 2019 and December 2020 and included 60 patients with β-TM diagnosed with hemoglobin electrophoresis. The control group included 30 healthy children matched for age and sex. All β-TM patients were on a regular blood transfusion program to maintain hemoglobin levels > 9 g%. Diagnosis of CKD was based on persistent albuminuria (albumin/creatinine > 30 mg/gm creatinine) ± eGFR < 90 mL/min/ 1.73m^2 for at least three months. eGFR was calculated according to the simple height–independent formula as follows (eGFR = 107.3 / (Scr/Q) where Q = 0.0270 × Age + 0.2329.[16] β-TM patients were divided into two groups: β-TM with CKD and β-TM without CKD. The exclusion criteria were: history of recurrent urinary tract infections, systemic diseases with affecting kidneys, use of nephrotoxic drugs, familial inherited kidney diseases, known associated chronic kidney, liver, heart, or endocrinal diseases, and history of any acute infection in preceding four weeks. All patients included in the study were subjected to detailed clinical evaluation, including history taking (age, age at the first blood transfusion., type of iron chelator, adherence to chelation therapy, splenectomy), thorough clinical examination with reporting of the anthropometric measures, and presence of organomegaly, laboratory investigations included complete blood counts, reticulocyte count, serum ferritin., serum creatinine, albumin/creatinine ratio in urine (ACR), serum glucose levels and plasma miRNA-451 expression level by real-time quantitative reversed transcription PCR.

Plasma RNA Extraction

Three milliliters of EDTA-preserved venous blood samples were centrifuged at 1600 xg at 4 degrees Celsius (C) for 20 minutes, plasma was extracted and stored at -80 °C until the assay time and miRNA-451 was extracted from plasma samples using the Qiagen miRNA Easy kit (Qiagen, Germany) according to the manufacturer's instructions to elute the RNA, including miRNA. A total of 200 µL of plasma was homogenized with 1000 µL of QIAzol lysis reagent using vortexing and pipetting and then incubated at room temperature for 20 minutes. The suspension was vigorously shaken before adding 200 µl of chloroform. The samples were centrifuged for 15 minutes at four °C at 12,000 g, and the RNA in the aqueous phase was precipitated for 10 minutes with 400 µL isopropanol at room temperature. The RNA pellets were re-solubilized in 30 µL RNase-free water after rinsing in 1 mL ethanol (70%). The A260/280 nm ratio was used to assess the integrity and concentration of RNA using the NanoDrop ND- 2000 UV spectrophotometry (Thermo Scientific, USA).

Real-time Quantitative Reversed Transcription PCR (RT-qPCR) Analysis

Using a TaqMan® MicroRNA Reverse Transcription kit, 5 µL of eluted RNA was transcribed to cDNA (Applied Biosystems; Thermo Fisher Scientific, Inc.). QPCR analysis was carried out using TaqMan microRNA assay kits and Universal PCR Master Mix (Applied Biosystems;

Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions using TM Step One TM to detect the miRNAs (Applied Biosystems, Thermo Fisher Scientific). The expression of a known quantity of miRNA RNU6B, an endogenous control, was used to normalize the RT-qPCR analysis of plasma miRNA-451 (Applied Biosystems; Thermo Fisher Scientific, Inc.). To analyze miRNA-451 expression in plasma, the Δ Cq value of miRNA-451 was calculated by subtracting the Cq of miRNA-451 from the average Cq of MiRNA RNU6B.

Statistical Analysis

All analyses were performed using SPSS, version 20. Comparisons of normally distributed continuous data were done by using the t tests, and values were expressed as mean ± standard deviation (SD). Chi-square test was used for comparison of categorical variables, and data were presented as frequency and percentage. Person correlations test was used to correlate quantitative variables fulfilling normal distribution. Logistic regression was used to examine the association between miRNA-451 expression and CKD risk. Receiver operating characteristic (ROC) curve was used to determine the best cutoff value of miRNA-451 that can discriminate between patients with and without CKD. A *P* value < .05 was considered to be statistically significant.

RESULTS

Sixty children, 36 males (60 %) and 24 females (40%), with β-TM were included in the study as the case and thirty healthy children, 22 males (73.3%) and 8 females (26.7%), as the control group. Chronic kidney disease (CKD) was reported in 24 patients (40%), and 14 patients (23%) were splenectomized (6 in the CKD group and 8 in the non-CKD group). The clinical and laboratory characteristics of the cases and controls are shown in Table 1. Serum creatinine, ACR and miRNA-451 expression levels were significantly higher in the case than in control group, while eGFR levels were significantly lower in cases than in controls. Comparing the patients with CKD and without CKD showed that, miRNA-451 levels were significantly lower in the CKD group (Table 2). The miRNA-451 was under-expressed in 16 β-TM patients (53.3%) with CKD compared to 4 patients (13.3%) without CKD ($P < .05$). The miRNA-451 expression levels were significantly higher in splenectomized patients (29.8 ± 12.42) than non-splenectomized patients (24.02 ± 8.26, $P < .05$). In splenectomized the miRNA-451 levels were significantly lower in patients with CKD compared to those without CKD ($P < .05$).

In univariate analysis, miRNA-451 expression levels had significantly positive correlation with reticulocyte counts (r = 0.27, $P < .05$), platelet count (r = 0.29, $P < .05$) and ferritin (r = 0.48, $P < .001$). Moreover, the eGFR had significantly positive correlation with miRNA-451 expression levels (r = 0.385, $P < .05$) and significantly negative correlation with reticulocytes counts (r = -0.296, $P < .05$), ferritin (r = -0.24, $P < .05$), and ACR (r = -.329,

Table 1. Comparison Between Controls and Patients as Regard Clinical and Laboratory Data

	Controls	Cases	*P*
Age, y	8.900 ± 2.594	9.866 ± 3.558	> .05
Height, cm	133.133 ± 11.270	123.266 ± 14.752	< .05
Weight, kg	24.000 ± 13.379	25.433 ± 7.659	> .05
Systolic BP, mmHg	92.000 ± 4.068	94.000 ± 6.689	> .05
Diastolic BP, mmHg	60.666 ± 2.537	62.000 ± 4.8113	> .05
TLC x 10^3 /cm^3	7.360 ± 1.897	8.750 ± 5.083	> .05
HB, gm%	11.813 ± 1.071	9.356 ± 0.804	< .001
PLT x 10^3 /cm^3	255.800 ± 52.490	296.366 ± 93.608	< .05
Ferritin, mg/dL	55.600 ± 29.134	1547.233 ± 791.535	< .001
Creatinine, mg/dL	0.326 ± 0.078	0.416 ± 0.154	< .05
Retics, %	0.766 ± 0.191	1.881 ± 0.545	< .001
Blood Glucose, mg/dL	95.800 ± 7.585	102.266 ± 19.561	> .05
eGFR, mL/min/ 1.73m^2	166.533 ± 41.397	144.766 ± 48.444	< .05
miRNA-451 Level	9.453 ± 5.753	25.326 ± 12.191	< .001
ACR, mg/gm Creatinine)	7.3 (4.4-15.6)	19.4 (3.9-733)	< .05

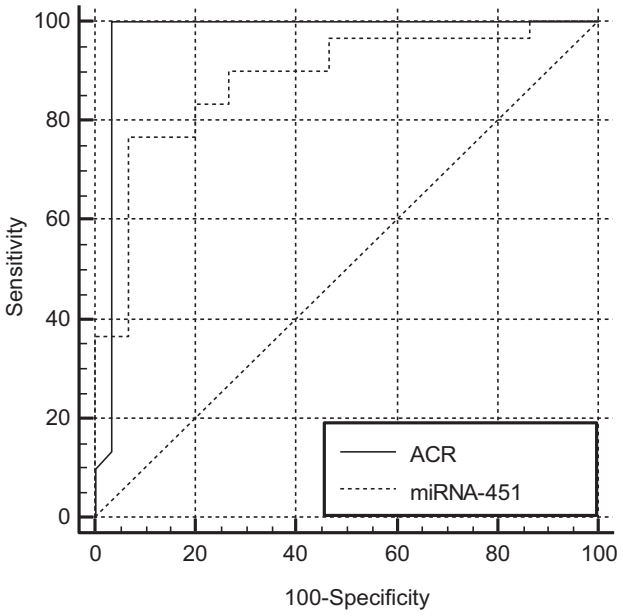
Abbreviations: BP, blood pressure; TLC, total leucocyte counts; PLT, platelets; Retics, reticulocytes; eGFR, estimated glomerular filtration rate; ACR, albumin / creatinine ratio.

Table 2. Comparison Between Patients With and Without CKD

	With CKD (n = 24)	Without CKD (n = 36)	*P*
Age, y	10.416 ± 4.031	9.500 ± 3.211	> .05
Age at 1st Transfusion, mo	6.333 ± 2.296	8.888 ± 0.974	> .05
Height, cm	132.666 ± 12.103	133.444 ± 10.675	> .05
Weight, kg	26.833 ± 9.182	24.500 ± 6.290	> .05
Transfusion Intervals, d	37.500 ± 13.269	36.666 ± 10.954	> .05
Systolic BP, mmHg	95.833 ± 7.755	92.777 ± 5.662	> .05
Diastolic BP, mmHg	63.333 ± 4.815	61.111 ± 4.646	> .05
TLC x 10^3 / cm^3	8.833 ± 4.124	8.694 ± 5.690	> .05
HB, gm%	9.558 ± 1.000	9.222 ± 0.621	> .05
PLT × 10^3 / cm^3	254.833 ± 72.261	324.055 ± 96.789	< .05
Ferritin, mg/dL	1425.416 ± 563.226	1628.444 ± 911.443	> .05
Creatinine, mg/dL	0.482 ± 0.155	0.372 ± 0.138	< .05
Blood Glucose, mg/dL	105.750 ± 15.632	99.944 ± 21.692	> .05
Retics, %	3.808 ± 0.6266	1.800 ± 0.268	< .001
ACR, mg/gm Creatinine	122.475 ± 19.285	16.085 ± 9.448	< .05
miRNA-451 Fold Change	14.834 ± 16.669	27.596 ± 13.437	< .05
eGFR, mL/min/ 1.73m^2	114.750 ± 30.331	164.77 ± 48.184	< .001

Abbreviations: BP, blood pressure; TLC, total leucocyte counts; PLT, platelets; Retics, reticulocytes; eGFR, estimated glomerular filtration rate; ACR, albumin / creatinine ratio.

P < .05). Linear logistic regression analysis showed that, low plasma microRNA-451 was a significant and independent predictor for CKD (B = -2.01, SE = 0.66, 95% CI: 1.54 to 2.21; *P* < .05). In ROC curve analysis, a cutoff value of ≤ 21 expression level could discriminate patients with CKD, with a sensitivity of 80% and specificity of 93.3 %, with 95% CI of 0.78 to 0.95, and AUC of 0.89. The comparison between the ROC curves for miRNA-451 and ACR was not statistically significant (Figure 1).



Comparison Between ROC Curves for miRNA-451 and ACR

DISCUSSION

Patients with β -thalassemia may have renal impairment in long-term follow-up. Chronic anemia, iron excess, and particular iron chelators are the known contributing factors.[17] In these patients, apoptosis and epithelial-mesenchymal transition occur in tubular cells with higher metabolic requirements, resulting in tubulointerstitial damage, glomerular sclerosis, and scarring.[18] Furthermore, iron overload-induced tubular cell injury can result in the migration of damaged cells into the interstitium. Tubulointerstitial scarring and glomerular sclerosis can occur due to release of cytokines and growth factors, resulting in decreased GFR.[19] In patients with β -TM hemosiderin deposits can be found in both the terminal section of the proximal tubules and in the distal tubules. Iron dissociates from transferrin in the acidic proximal tubular fluid, and induces the generation of reactive oxygen species with consequent override of the cellular antioxidative defense mechanisms and damage to the brush border of the renal tubular cells.[20] Oxidative stress exacerbates intravascular and extravascular hemolysis, inefficient erythropoiesis, and causes major organ failure, mostly the heart, liver, endocrine system, and kidneys.[21] Oxidative stress is common in erythroid cells while, they have several defense mechanisms, including FoxO3-regulated antioxidant enzymes.[22] It is reported that, miRNA-451 have a protective role against

oxidative stress. In animal research, deletion of genomic miRNA-451 regions elevates the level of miRNA-451 target gene (Ywhaz) product, 14–3-3 zeta, which repossesses the nuclear factor FoxO3 in the cytoplasm, preventing FoxO3 dependent transcription of catalase (Cat) and glutathione peroxidase 1 (GPx1) antioxidant enzymes.[23] According to prior research, oxidative stress is the mutual connection between the key pathways, responsible for the development of diabetic micro- and macrovascular complications. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, advanced glycation end products (AGE), polyol pathway abnormalities, uncoupled nitric oxide synthase (NOS), and mitochondrial respiratory chain through oxidative phosphorylation are among the macromolecules linked to enhanced reactive oxygen species (ROS) generation. Excess ROS affects protein kinase C, mitogen-activated protein kinases, and various cytokines and transcription factors, leading to increased extracellular matrix expression (ECM).[24] Zhang *et al.*[15] evaluated the role of miRNA-451 in mesangial hypertrophy during early diabetic nephropathy in a T2DM mouse model, in vitro. They detected five probable miRNA-451 target genes that reduce the expression of the Ywhaz (tyrosine 3-monooxygenase, tryptophan 5-monooxygenase activation protein, zeta) gene. Overexpression of miRNA-451 inhibits Ywhaz and decreases the activity of two kinases (p38 MAPK and MKK3). Targeting the Ywhaz gene decreases mesangial hypertrophy and glomerular mesangial cell proliferation in vitro and vivo. The urine albumin/creatinine ratio and eGFR were used to track the course of chronic kidney disease.[25, 26] However, in terms of sensitivity and effectiveness, in identifying renal impairment, these parameters have some limitations. The goal of this study was to explore the link between miRNA-451 expression and chronic kidney disease in children with β -TM. Our findings demonstrated that patients with β -TM had higher levels of miRNA-451 expression than healthy controls. In addition, splenectomized patients had significantly higher miRNA-451 expression levels. Leecharoenkiat *et al* discovered that patients with β -thalassemia have higher plasma miRNA-451 levels, consistent with our findings.[14] Furthermore, splenectomized patients had significantly higher miRNA-451 levels than non-splenectomized patients. They also revealed that higher miRNA-451 levels were substantially linked to reticulocyte and platelet counts, confirming our findings. Previously miRNA-451 was discovered to be up-regulated in a lineage-specific manner during early erythropoiesis during erythroid series maturation.[27] In patients with β -TM, inadequate erythropoiesis and hemolysis cause the release of intracellular miRNA-451 from hemolyzed RBCs into the bloodstream, therefore increased erythropoiesis by the bone marrow, results in reticulocytosis and a rise in miRNA-451 levels.[28] In the current study, we found that the levels of miRNA-451 expression were significantly lower in patients with CKD than those without CKD. Estimated GFR was also found to have a strong positive correlation with miRNA-451, and a significant negative correlation with ACR. In the linear logistic regression analysis, low plasma microRNA-451 levels were significant independent predictors of CKD. Muendlein *et al.* published a study that found significant positive correlations between miRNA-451 levels and eGFR in CKD patients ($r \sim 0.361$, $P \sim .001$).[29] They also discovered that miRNA-451 and ACR have a significant negative relationship ($r \sim 0.129$, $P < .05$). We found a strong negative correlation between eGFR and reticulocyte counts, implying that the severity of hemolysis and anemia affect the progression of CKD in patients with β -TM. We also investigated the levels of miRNA-451 expression in the two study groups of splenectomized patients, to see if splenectomy had affected miRNA-451 expression levels, and found that the levels were still considerably lower in splenectomized patients with CKD compared to those without CKD. The levels of miRNA-451 expression were observed to be lower in plasma and urine of patients with stage five CKD compared to CKD stage three in adults with diabetic nephropathy, suggesting that serum miRNA-451 could be used as a predictor of disease progression, according to Sayilar *et al.*[30] As we didn't measure urine levels of miRNA, we could not determine why plasma levels of miRNA-451 were low in these patients. Nonetheless, a recent study reported lower levels of miRNA-451 in the urine of CKD patients [30]. In contrast, another study found that loss of miRNA-451 in the proximal tubules is associated with significantly higher urinary exosomes miRNA-451 expression in diabetic and nondiabetic CKD than in healthy controls, with simultaneous activation of the YWHAZ and

CAB39 genes and low renal miRNA-451 levels.[31] Our study found that a cutoff value of 21 for miRNA-451 expression level may distinguish between individuals with and without CKD. Xiao *et al.* conducted their research on plasma miRNAs profile in patients with focal segmental glomerular sclerosis (FSGS) and reported down-regulated expression of miRNA-451 in patients with more severe FSGS.[32] They also found that miRNA-451 had an AUC of 0.76 (95% CI: 0.67 to 0.85), by ROC analysis, similar to our findings. Although urine albumin excretion (UAE) is a convenient test to detect early CKD, there are sometimes contradictions between the ACR and 24h UAE. Additionally, some studies have reported the progression of CKD in diabetic kidney disease in the absence of albuminuria, making the prediction of CKD progression in patients with normal UAE values and maintained (> 60 mL/min/ 1.73m^2) eGFR very challenging. To the best of our knowledge, this is the first study to investigate the role of miRNA-451 in developing CKD in pediatric β-TM.

Limitations of the stud were the relatively small sample size and lack of measurement of urinary levels of miRNA-451.

CONCLUSION

CKD is a problem of rising concern in patients with β-TM. Low plasma expression of miRNA-451 is associated with development CKD in children suffering β-TM

COMPLIANCE WITH ETHICAL STANDARDS

- The authors declare that there is no conflict of interest.
- An informed consent was obtained from patients' caregivers
- University hospital ethical committee approved the research protocol.

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