Original Article

DOI: 10.7860/JCDR/2023/59329.17683

Serum miRNA-30a-5p in Steroid Sensitive Idiopathic Nephrotic Syndrome in Indian Children: A Case-control Study

Paediatrics Section

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ABSTRACT

Introduction: Childhood Nephrotic Syndrome (NS) is a podocytopathy. Micro Ribonucleic Acid (miRNA), composed of 21-25 non coding nucleotides, regulates gene expression by inhibiting protein transcription by binding to complementary messenger RNA. The microRNA-30a is expressed in the human glomerular podocytes and collecting ducts. This microRNA protects the podocytes by targeting the Calcineurin-nuclear Factor of Activated T cells (NFATc) pathways. Serum microRNA-30a-5p is a validated biomarker which is upregulated in NS.

Aim: To determine the serum miRNA-30a-5p in steroid sensitive idiopathic NS in Indian children.

Materials and Methods: This case-control study was conducted at the Department of Paediatrics, KS Hegde Medical Academy, Mangalore, Karnataka, India, from from January 2018 to June 2019. Thirty children with NS and age and gender matched controls were recruited. Relative expression of microRNA-30a-5p was analysed by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR). Estimations were done both in cases and controls at enrollment and also at four weeks when in remission in cases. The fold change was calculated as a power of cycle threshold. Statistical tests Kolmogorov-Smirnov test was used to establish the normality using Statistical Package for Social Sciences (SPSS) version 22.0.

Results: There was upregulation of microRNA-30a-5p expression among children with NS with a significant fold change (~184) at enrollment. The levels declined, but remained above baseline (~6) after four weeks of treatment when compared to controls. The mean differences in delta threshold cycle and threshold cycle between the three groups were significant ($p<0.001$). There was no correlation with the biochemical parameters.

Conclusion: The present study concludes that serum microRNA-30a-5p expression is upregulated in children with steroid sensitive NS in Indian children.

Keywords: Delta threshold cycle, Micro ribonucleic acid, Podocytopathy, Prednisolone therapy

INTRODUCTION

Childhood NS is a common glomerular disease characterised by massive selective proteinuria. Grouped under podocytopathy, immune dysregulation and systemic circulating factors are a few proposed mechanisms for the podocyte injury seen in primary idiopathic NS [1,2]. The disorder is further classified as minimal change disease, focal segmental glomerulosclerosis, and membranous nephropathy by histopathology, and steroid-sensitive and steroid-resistant by treatment response to prednisolone. Relapses occur in two-thirds of children [2].

Studies explore novel non invasive biomarkers to detect and monitor disease activity in renal diseases in children [3-5]. Nearly one-third of the genome is estimated to be regulated by around 2000 miRNAs so far identified [6]. The miRNAs, composed of 21-25 non coding nucleotides bind to complementary messenger RNAs and inhibit protein transcription [7]. Thus, they are important in gene expression. The miRNA-30a is expressed in the human glomerular podocytes and collecting ducts [8]. In mouse models, this miRNA maintains the normal podocyte morphology by targeting the calcineurin-nuclear factor of activated T cells (NFATc) pathways [8-10]. The dysregulation of miRNAs results in renal injury. In children with NS, the most scrutinised miRNA is serum miRNA-30a-5p and is a validated biomarker that is upregulated in NS [11-13].

There are no Indian studies on miRNA expression in NS, and hence this study on miRNA-30a-5p expression in children with steroid sensitive NS. The children were evaluated for microRNA-30a-5p on enrollment and four weeks into prednisolone therapy in remission using RT-qPCR. The authors hypothesised an elevation in serum miRNA-30a-5p by several folds during disease state with a return to baseline after four weeks of prednisolone therapy.

MATERIALS AND METHODS

A case-control study was conducted at the Department of Paediatrics, KS Hegde Medical Academy, Mangalore, Karanataka, India, from January 2018 to June 2019. Institutional Ethical Committee clearance (INST.EC/EC/075/2017-18) and written informed parental consent from all enrolled were obtained.

Sample size calculation: Sample size was determined using www.openepi.com/SampleSize/SSCC.htm assuming 95% Confidence Interval (CI) and 80% power.

Inclusion criteria: Thirty children aged between one and 18 years diagnosed as idiopathic NS were enrolled as cases either at the first episode or relapse. An equal number of healthy children, age and gender-matched were the controls.

Exclusion criteria: Congenital, infantile, secondary, and steroid resistant NS were excluded. Children with NS presenting with infections, idiopathic NS on immunosuppressant therapy other than prednisolone were also excluded.

Study Procedure

Demographic data included age, gender, clinical and lab status of NS, atypical features, and response to prednisolone therapy. Standard case definitions were used [2]. The first episode of NS was diagnosed in the presence of hypoalbuminaemia (≤2.5 g/dL), oedema, hyperlipidaemia (cholesterol >200 mg/dL) with spot urine protein and creatinine ratio >2, and early morning urine protein ≥3+ on the dipstick. Relapse was defined as urine albumin ≥3+ in three consecutive early morning specimens, having been in remission previously. Remission was defined as urine albumin nil or trace in three consecutive early morning specimens. Frequent relapsers were those with ≥2 relapses in initial six months or ≥4 in a year [14].

The authors estimated the relative miRNA-30a-5p expression by RT-qPCR using glyceraldehyde 3-phosphate dehydrogenase as the reference gene. Five millilitres of blood was obtained from the cases (pretreatment group) and controls at enrollment. A second sample was obtained after four weeks only from the cases (treatment group) when in remission. The serum was separated by centrifuging at 20,000 g for 10 minutes and stored at -80°C until further processing. RNA isolation, complementary de-oxy RNA synthesis, and amplification of miRNA-30a-5p were carried out sequentially using commercial kits according to the instructions given with the kit (mirVana™ PARIS™ RNA and native protein purification kit, TaqMan® advanced miRNA cDNA synthesis kit, and TaqMan® Advanced miRNA Assays from Applied Biosystems, Thermo Fisher Scientific).

The miRNA-30a-5p expression was normalised to the reference gene by calculating the delta threshold cycle (ΔCt) and further relative to the healthy controls by delta delta threshold cycle (ΔΔCt) [15]. The fold change was calculated by $2^{-\Delta\Delta Ct}$ and corrected for a ratio [16]. In the present study, miRNA was considered upregulated, if there was atleast a 1.5-fold increase in NS than the control, and Ct was <35 in the control sample [11].

STATISTICAL ANALYSIS

Data were analysed using IBM SPSS, USA. The Kolmogorov-Smirnov test was used to establish the normality of the ΔCt data. In children with NS, miRNA-30a-5p expression was compared using the paired t-test. The comparison between the cases and the controls was made by an Independent sample t-test. A p<0.05 was considered to be significant.

RESULTS

A total of 60 children were recruited in the study, 30 cases of children with NS and 30 controls. The study group's median age was seven years, with 1.03:1 male and female distribution. Sixteen (53.3%) children were included during a relapse of which seven were frequent relapsers. Atypical features like haematuria and hypertension were seen in 3.3% and 6.7% of cases, respectively [Table/Fig-1]. The biochemical characteristics of the children with NS are mentioned in [Table/Fig-2]. The miRNA-30a-5p expression is given in [Table/Fig-3]. The results showed upregulation of the miRNA-30a-5p at enrollment. The levels declined but remained above baseline after four weeks of treatment in children with NS [Table/Fig-3]. The magnitude of fold change was ~184 at enrollment, and ~6 four weeks into treatment in comparison to control. The mean differences in delta threshold cycle and threshold cycle between the three groups were significant [Table/Fig-4]. There was no correlation between miRNA expression with urine protein creatinine ratio, serum albumin, or serum cholesterol. Comparison of miRNA expression within the case group, namely, between the first episode and relapse and frequent and infrequent relapses, did not show statistical significance.

Characteristics	Cases n=30 (%)	Controls n=30 (%)
Age (in years)		
1-10	20 (66%)	20 (66%)
11-18	10 (34%)	10 (34%)
Gender		
Male	17 (57%)	17 (57%)
Female	13 (44%)	13 (44%)
Presentation		
First Episode	14 (47%)	-
Relapse	16 (53%)	-
Atypical features		
Haematuria	1 (3.3%)	-
Hypertension	2 (6.7%)	-
Frequent relapse	7 (43.7%)	-
Infrequent relapse	9 (56.3%)	-
Treatment response to prednisolone		
Steroid sensitive	24 (80%)	-
Steroid dependent	6 (20%)	-

[Table/Fig-1]: Demographic and baseline characteristics of study population.

Biochemical parameters	Median (25th centile, 75th centile) n=30
Serum albumin (mg/dL)	1.6 (1.25, 1.90)
Serum cholesterol (mg/dL)	448.0 (254.5, 526.0)
Serum triglyceride (mg/dL)	309.0 (209.0, 676.0)

[Table/Fig-2]: Biochemical characteristics of the study population.

MiRNA-30a-5p expression	Nephrotic syndrome		Control
	Pretreatment baseline levels	After 4 weeks of treatment	
Delta threshold cycle (ΔCt)	15.8 (2.1)	10.9 (1.6)	8.6 (1.1)
Fold change ($2^{-\Delta\Delta Ct}$)	323.6 (457.7)	9.5 (11.8)	1.3 (0.8)
Corrected fold change	183.6	5.8	1.0

[Table/Fig-3]: MiRNA-30a-5p expression in the study group.
Data presented as mean (SD)

Comparison groups	Mean difference (95% Confidence interval)		Statistics
	Delta threshold cycle (ΔCt)	Fold change (2-ΔΔCt corrected)	p-value
Pretreatment and control[a]	7.2 (6.8, 7.6)	182.6 (134.3, 231.0)	<0.001
Treatment and control[a]	2.4 (2.1, 2.6)	4.8 (3.6, 5.9)	<0.001
Pretreatment and treatment[b]	4.8 (4.5, 5.1)	177.9 (130.6, 225.1)	<0.001

[Table/Fig-4]: Comparison of miRNA-30a-5p expression between the study groups.
[a]Independent sample t-test; [b]paired t-test

DISCUSSION

The present study examined the relative expression of previously validated serum miRNA-30a-5p in idiopathic NS pretreatment and after four weeks using RT-qPCR assay in an Indian population. The results showed upregulation among cases. The enrollment cases had a significant fold change (~184) as calculated by the comparative Ct method. Despite a marked decline, the values four weeks into treatment were significantly higher (~6) compared to controls. The miRNA-30a-5p is shown to be abundant in human glomerular podocytes [8] and is protective against injury to them [17].

According to study by Luo Y et al., the concentrations of serum and urinary miR-30a-5p, were significantly increased in NS children compared with controls and the concentrations markedly declined with the clinical improvement [11]. Similar observations were made by Zhang W et al., and Teng J et al. Thus, it is a validated biomarker in NS in Chinese ethnicity [11-13]. Wu J et al., demonstrated the downregulation of miRNA-30a-5p by in-situ hybridisation in the sclerotic areas of FSGS renal biopsy specimens. On the basis of their findings, following treatment of podocytes with transforming growth factor beta, lipopolysaccharide etc. in the presence or absence of glucocorticoids, found that glucocorticoids prevented downregulation of miR-30 [17].

Luo Y et al., demonstrated a 2.5-fold change in miRNA-30a-5p at enrollment, compared to controls (p=0.001). The values returned to normal after four weeks of steroid therapy. The triglycerides had a negative correlation. The miRNA expression was similar in the steroid-sensitive and steroid-resistant groups [11]. Teng J et al., showed similar results in their group of patients with NS with significantly overexpressed miRNA-30a of 2.6-fold change in NS patients. The expression in the steroid-resistant was significantly higher than the steroid-sensitive NS [13]. Zhang W et al., showed

elevated urine miRNA-30a-5p excretion in patients with Focal Segmental Glomerulosclerosis (FSGS) [12]. These authors screened several miRNAs of clinical interest by low-density microarray and validated each of them, including miRNA-30a-5p, by absolute quantification using RT-qPCR [11-13].

Limitation(s)

The present study was a preliminary study, to look into the trend of miRNA-30a-5p in children with steroid sensitive NS. The present study did not ascertain treatment response across various categories of NS.

CONCLUSION(S)

The present study established an upregulation of miRNA-30a-5p in the children with steroid sensitive NS. The expression of miRNA-30a-5p was higher than in the control group. The study suggested that miRNA-30a-5p plays an essential role in the progression of NS and has the potential as a novel biomarker of childhood NS. Further studies on miRNA-30a-5p in NS are required, to explore the mechanism of its influence on NS and its pathological types.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jul 28, 2022
- Manual Googling: Nov 30, 2022
- iThenticate Software: Dec 10, 2022 (9%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jul 27, 2022**
Date of Peer Review: **Oct 27, 2022**
Date of Acceptance: **Dec 14, 2022**
Date of Publishing: **Mar 01, 2023**