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Original Article

LMX1B gene polymorphisms in Chinese children with idiopathic nephrotic syndrome

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Abstract: The LIM homeobox transcription factor 1, beta (LMX1B) gene is associated with steroid-resistant nephrotic syndrome and plays a crucial role in regulating other resistant genes, such as CD2AP, NPHS2, NPHS1, ACTN4, COL4A3 and COL4A4. Therefore, we aimed to investigate whether LMX1B gene variation might be a predictor for steroid resistance in pediatric patients with nephrotic syndrome. Two SNPs (rs34682917 and rs13295990) in the LMX1B gene were genotyped using DNA Direct PCR-sequencing method in 74 controls and 104 pediatric patients with INS, of whom were 53 steroid-sensitive (SS) cases and 51 steroid-resistant (SR) cases. The frequencies of the LMX1B rs34682917 TT (25.5 vs 3.8%) genotype or T allele (49 vs 31.1%) and rs13295990 CC (43.1 vs 24.5%) or C allele (67.6 vs 53.8%) were significantly higher in SR subjects than in SS subjects. Analysis of LMX1B haplotypes discovered that the frequency of the T-C haplotype was significantly higher in SR patients than in SS patients (49 vs 31.1%). There was no association between the LMX1B gene polymorphisms or haplotype and onset of INS. To conclude, these results indicate that genetic variations in the LMX1B gene are risk factors for increased steroid resistance in INS patients.

Keywords: Childhood nephrotic syndrome, steroid, LIM homeobox transcription factor 1, beta, polymorphism

Introduction

Idiopathic nephrotic syndrome (INS), which is characterized by edema, proteinuria, hypoalbuminemia and hyperlipidemia, is one of the most common primary glomerular diseases that occur in children. It can be clinically categorized by the responsiveness to oral steroid therapy as steroid sensitive (SS) or steroid resistant (SR). Steroid responsiveness is the major prognostic indicator of this disease [1]. However, the exact mechanism for steroid resistance in INS patients is still unclear.

The LIM homeobox transcription factor 1, beta (LMX1B) gene, mapped on chromosome 9q33, consists of eight exons and encodes a LIM-homeodomain transcription factor which contains two zinc-binding LIM domains, a DNA-binding homeodomain and a glutamine-rich domain [2, 3]. The LIM domains involved in protein-protein interactions are encoded by exons2-3, and the homeodomain, which is necessary for transcriptional activation and DNA binding, is encoded by exons4-6 [2, 4, 5].

Recent studies have indicated that the LMX1B gene may perform a pathogenic role in renal diseases [6-9]. LMX1B is expressed in the kidney, especially in podocytes, and pivotal in forming foot processes and slit diaphragms [4]. Endele et al. observed that Lmx1b knockout mice present a significantly lower increase in glomerular volume and an increase in glomerulosclerosis [10]. Later, Zhou et al. revealed that LMX1B affects typical markers of fibrosis and reduced LMX1B levels result in renal fibrosis [11, 12]. Clinically, 30% patients with nail-patella syndrome (NPS) caused by LMX1B mutations present with nephropathy, including nephrotic syndrome in children [13-18]. Furthermore, Lee et al. reported that an NPS patient with nephrotic syndrome at age 2.2 show steroid resistant to oral steroid therapy and rapidly progress to end-stage renal disease [13]. The LMX1B gene has been identified as one of numerous resistant genes [19]. Some researchers have manifested that LMX1B regulates expression of CD2AP, NPHS2, NPHS1, ACTN4, COL4A3 and COL4A4 [4, 8, 20-22], which are associated

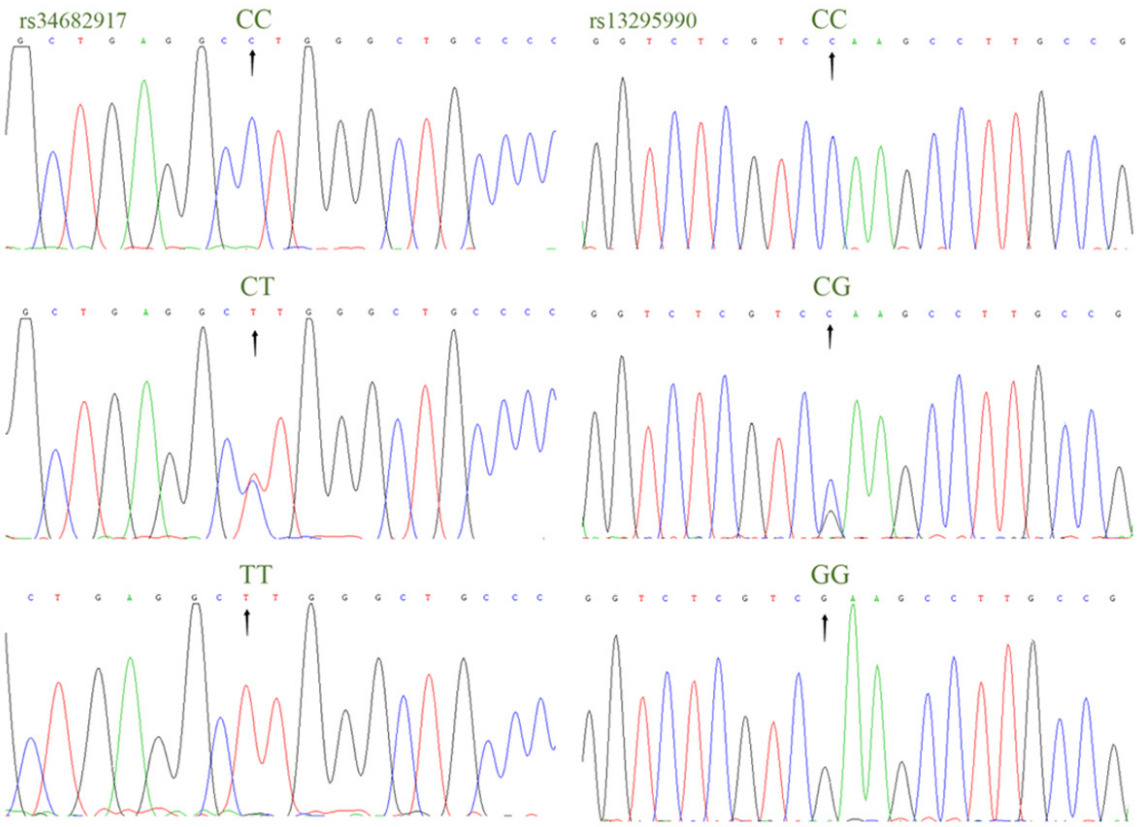

Figure 1. Sequencing analysis of LMX1B gene. The genotypes of rs34682917 and rs13295990.

Table 1. Demographic profiles of patients

Parameter	*SS*	*SR*
Patients (n)	53	51
Gender (n)		
Male	44	35
Female	9	16
Age of onset [median (range)]	4 (1-13)	6 (1-14)
Renal biopsy (n)	11	15

SS, steroid sensitive; SR, steroid resistant.

with steroid-resistant nephrotic syndrome [19]. In addition, making early diagnosis of patients with steroid resistance is difficult due to numerous resistant genes. Therefore, we hypothesized that LMX1B gene variation might serve as a predictor for early diagnosis in patients with steroid resistance.

As far as we know there are no previous studies investigating the role of LMX1B gene in childhood nephrotic syndrome among Chinese population. Moreover, considering that gene variation located in LMX1B homedomain interacting with other resistant genes [2, 5] is closely associated with kidney disease [14, 23], we aimed to sequence directly exons4-6 to explore whether LMX1B gene variation is associated with risk of INS, and to investigate whether they influence patient's initial steroid responsiveness in Chinese children.

Patients and methods

Patients and controls

One hundred and four Chinese patients with INS who were admitted to the Department of Pediatrics, The First Affiliated Hospital of Guangxi Medical University during the period January 2010 to February 2014 were recruited to our study. INS patients were diagnosed according to the criteria of the International Study of Kidney Disease in Children, including massive proteinuria of 40 mg/h/m^2 and hypoalbuminemia of ≤2.5 g/dl. All the patients received standard initial steroid therapy and were categorized by their initial response to steroid treatment as steroid sensitive (SS) or ste-

Table 2. Distributions of genotypes and alleles in patients and control subjects

Single nucleotide polymorphism	Genotype	Patient group (n=104)	Control group (n=74)	OR (95% CI)	*P* value
rs34682917	CC	36 (34.6%)	26 (35.1%)	1 (reference)	
	CT	53 (51%)	35 (47.3%)	1.07 (0.55-2.09)	0.844
	TT	15 (14.4%)	13 (17.6%)	0.83 (0.34-2.04)	0.680
	C allele	125 (60.1%)	87 (58.8%)	1 (reference)	
	T allele	83 (39.9%)	61 (41.2%)	0.94 (0.61-1.45)	0.776
rs13295990	GG	13 (12.5%)	8 (10.8%)	1 (reference)	
	GC	56 (53.8%)	39 (52.7%)	0.86 (0.32-2.28)	0.757
	CC	35 (33.7%)	27 (36.5%)	0.76 (0.27-2.13)	0.598
	G allele	82 (39.4%)	55 (37.2%)	1 (reference)	
	C allele	126 (60.6%)	93 (62.8%)	0.89 (0.58-1.38)	0.610

Table 3. Distributions of genotypes and alleles among patients with respect to age of onset

Single nucleotide polymorphism	Genotype	Age of onset ≥6 years (n=41)	Age of onset <6 years (n=63)	OR (95% CI)	*P* value
rs34682917	CC	16 (39%)	19 (30.2%)	1 (reference)	
	CT	20 (48.8%)	33 (52.4%)	1.23 (0.51-2.94)	0.649
	TT	5 (12.2%)	11 (17.5%)	1.57 (0.44-5.62)	0.485
	C allele	52 (63.4%)	71 (56.3%)	1 (reference)	
	T allele	30 (36.6%)	55 (43.7%)	1.23 (0.69-2.19)	0.482
rs13295990	GG	7 (17.1%)	6 (9.5%)	1 (reference)	
	GC	24 (58.5%)	32 (50.8%)	1.47 (0.43-5.00)	0.540
	CC	10 (24.4%)	25 (39.7%)	2.58 (0.68-9.82)	0.165
	G allele	38 (46.3%)	44 (34.9%)	1 (reference)	
	C allele	44 (53.7%)	82 (65.1%)	1.52 (0.85-2.70)	0.157

roid resistant (SR) after the first 4 weeks of full dose of prednisone therapy (2 mg/kg/day). SS was defined as the disappearance of proteinuria (negative to track in a urine strip for 3 consecutive days, or a urine protein/creatinine level of <0.2) while SR was defined as the persistence of proteinuria. The control group consisted of 74 Chinese healthy children with no history of kidney disease. The Ethics Committee of our Medical Faculty approved this study and informed consent was obtained from all subjects included in our study.

DNA extraction and genotyping

Genomic DNA was extracted using BloodGen Mini Kit (Cwbiotech, Beijing, China) following the manufacturer's instructions. DNA was stored at -20°C until needed. Genotyping for the LMX1B gene was performed by sequencing of PCR products. PCR amplification with the pair of primers (the forward primer 5-CCACGGCAGGTGTCAACAGA-3 and the reverse primer 5-GATGGCCTTGGTGGAAGGCT-3) [24] was performed in 50 μL reaction volumes that contained 100 ng genomic DNA, 2× GC buffer, 2.5 μM dNTP Mixture, and 0.25 U TaqDNA polymerase (Takara, Dalian, China). After an initial denaturation at 95°C for 10 min, amplification was performed 35 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The PCR products were sent to BGI Tech (Shenzhen, China) for sequencing. Finally, Two SNPs, rs34682917 and rs13295990, were genotyped in both controls and patients (**Figure 1**). Moreover, information about the functional effects of SNPs was obtained from F-SNP database (http://compbio.cs.queensu.ca/F-SNP/).

Statistical analyses

The Hardy-Weinberg equilibrium (HWE) assumption was performed with the Court online calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calcu-

Table 4. Distributions of genotypes and alleles in SR and SS patients

Single nucleotide polymorphism	Genotype	SS (n=53)	SR (n=51)	OR (95% CI)	*P* value
rs34682917	CC	22 (41.5%)	14 (27.5%)	1 (reference)	
	CT	29 (54.7%)	24 (47%)	1.49 (0.60-3.68)	0.392
	TT	2 (3.8%)	13 (25.5%)	13.23 (2.46-71.10)	0.003
	C allele	73 (68.9%)	52 (51%)	1 (reference)	
	T allele	33 (31.1%)	50 (49%)	2.39 (1.33-4.30)	0.004
rs13295990	GG	9 (17%)	4 (7.8%)	1 (reference)	
	GC	31 (58.5%)	25 (49%)	2.26 (0.56-9.11)	0.250
	CC	13 (24.5%)	22 (43.1%)	6.52 (1.43-29.70)	0.015
	G allele	49 (46.2%)	33 (32.4%)	1 (reference)	
	C allele	57 (53.8%)	69 (67.6%)	2.19 (1.21-3.99)	0.010

Table 5. Comparison of haplotype distributions in INS patients and controls

Haplotype	Haplotype frequency		Hap-score	*P*-value
	Controls	Patients		
T-C	0.41216	0.39904	0.97407	0.33002
C-C	0.21622	0.20673	-0.07107	0.94335
C-G	0.37162	0.39423	-1.03738	0.29956

Global Score Statistics: global-stat =1.22241, df =2, *p*-value =0.5427. Order of markers: rs34682917, rs13295990.

lator.xls) [25]. To compare continuous variables with abnormal distributions, the Mann-Whitney U test was used. Odds ratios (ORs) and confidence intervals (CIs) were calculated by logistic regression analysis adjusted for age and sex. A value of P <0.05 was regarded as statistically significant. Statistical analyses were performed via SPSS ver. 16 software excepting for haplotype analysis which was made using the R-program (ver2.10.0, 2009; The R Foundation for Statistical Computing) with the package haplo.stats.

Results

Demographic profiles of the participants

Demographic profiles of these INS patients were presented in **Table 1**. One hundred and four INS children (79 boys and 25 girls) with a median age of 5 (rang 1-14) years were enrolled in our study. The renal biopsy results with definitive diagnosis were available only for 26 cases including 12 (46.2%) minimal-change disease, 8 (30.8%) focal segmental glomerulosclerosis and 6 (23.1%) mesangial proliferative glomerulonephritis because some parents disagreed with kidney biopsy. The control group was comprised of 74 healthy children (54 boys and 20 girls) with a median age of 5 (rang 3-15) years.

Influence of LMX1B polymorphisms on the NS onset in patients

The genotype distributions of LMX1B polymorphisms did not deviate from Hardy-Weinberg equilibrium in controls and INS patients. Genotype and allele frequencies of LMX1B gene polymorphisms between the patients and healthy children were shown in **Table 2**. There were no significant differences in genotypes and allele distribution of LMX1B polymorphisms between INS patients and healthy children. The onset age of INS was not affected by any genotype or allele distribution in either SNP (**Table 3**).

Influence of LMX1B polymorphisms on initial steroid responsiveness

The genotypes and allele frequencies of rs34682917 and rs13295990 in INS patients were determined with respect to their initial response to prednisone (**Table 4**). The frequencies of the LMX1B rs34682917 TT (25.5 vs 3.8%; OR 13.23, 95% CI 2.46-71.10, P=0.003) genotype and T allele (49 vs 31.1%; OR 2.39, 95% CI 1.33-4.30, P=0.004) were significantly higher in SR subjects than in SS subjects. Similarly, patients carrying LMX1B 13295990 CC (43.1 vs 24.5%; OR 6.52, 95% CI 1.43-29.70, P=0.015) genotype and C (67.6 vs 53.8%; OR 2.19, 95% CI 1.21-3.99, P=0.010) allele had higher risks to develop steroid resistance.

Haplotype analysis

Haplotype analysis of two LMX1B polymorphisms (rs34682917 and rs13295990) in complete linkage disequilibrium (D'=1) revealed

Table 6. Comparison of haplotype distributions in INS patients with respect to age of onset

Haplotype	Haplotype frequency (age of onset)		Hap-score	*P*-value
	≥6 years	<6 years		
T-C	0.36585	0.42063	0.72552	0.46813
C-C	0.17073	0.23016	0.93001	0.35236
C-G	0.46341	0.34921	-1.53336	0.12519

Global Score Statistics: global-stat =2.5375, df =2, *p*-value =0.28118. Order of markers: rs34682917, rs13295990.

Table 7. Comparison of haplotype distributions in INS patients with SR and SS

Haplotype	Haplotype frequency		Hap-score	*P*-value
	SS	SR		
T-C	0.31132	0.4902	2.25467	0.02415
C-C	0.22642	0.18627	-1.19047	0.23386
C-G	0.46226	0.32353	-1.4622	0.14369

Global Score Statistics: global-stat =5.26499, df =2, *p*-value =0.0719. Order of markers: rs34682917, rs13295990.

three haplotypes (T-C, C-C and C-G). There were no differences in the distribution of LMX1B haplotypes between INS patients and the healthy children (**Table 5**). Also, it did not differ significantly in regard to age of onset (**Table 6**). However, the frequency of the T-C haplotype (49.0 vs 31.1%, hap-score =2.25, P=0.02) was significantly higher in patients with SR than among patients with SS (**Table 7**).

Discussion

In this study, two SNPs were genotyped in the LMX1B gene in pediatric patients with INS to explore the correlation between the genotypes/allele distributions and initial steroid responsiveness. The frequency distribution of LMX1B rs34682917 TT genotype and T allele were significantly higher in pediatric patients with SR than those with SS. The presence of LMX1B rs13295990 CC genotype and C allele increased the risk of developing corticosteroids resistance in INS patients. Furthermore, patients carrying the T-C haplotype had an enlarged risk of developing steroids resistance.

LMX1B plays an essential role in the development of diverse organs and tissue structures including limbs, brain, eyes and kidneys. Its pivotal function in the development of glomerulus has been noted. Many studies have observed that irregular thickening of the glomerular basement membrane (GBM) with occasional regions of membrane discontinuity and anomalous podocytes deficiency in forming foot processes and slit diaphragms occurred in the Lmx1b knockout mice kidneys [4, 26]. Endele et al. [10] observed a significantly lower increase in glomerular volume and an increase in glomerulosclerosis in Lmx1b knockout mice. The dysfunction of the podocyte foot processes and slit diaphragms can provide a better comprehension of the presented proteinuria and poor response to corticosteroid treatment. Furthermore, LMX1B can interact with these resistant genes, such as CD2AP, NPHS2, NPHS1, ACTN4, COL4A3 and COL4A4 [4, 8, 20-22], as their podocyte-specific upstream regulator through binding to the FLAT elements [27, 28]. Clinically, Boyer et al. [9] reported that LMX1B mutations in exon4, which would be expected to weaken the interaction between the homeodomain and DNA in silico homology model, can result in FSGS without extrarenal manifestations. Moreover, together with INF2 and WT1, LMX1B has been added to the list of switch-hitting genes that may lead to both syndromic and nonsyndromic FSGS by JASN [8]. Therefore, it's necessary to investigate the association between the LMX1B gene variation and steroid resistance in INS patients.

The higher frequency of rs34682917 polymorphism in the LMX1B gene in patients with SR compared to those with SS manifested that it may be a predictor for susceptibility to developing resistance to steroids. The real mechanism of the LMX1B gene in SR subjects is not identified. The LMX1B rs34682917 polymorphism is a non-coding intron variant, which is located in Intron 3. However, it may affect DNA transcription, alter splicing, RNA stability or be linked to other causal polymorphisms within the LMX1B gene [29, 30]. No functional information of rs34682917 was predicted from F-SNP database. Consequently, the possible reason may be being linked to rs13295990 polymorphism since patients with T-C haplotype were prone to developing steroids resistance.

It was also discovered that the frequency distribution of genotype or allele of LMX1B gene rs13295990 is significantly higher in pediatric patients with SR compared to those with SS.

The rs13295990 polymorphism, located in Exon 4 of the LMX1B gene, is a synonymous variant resulting in no amino acid changes (Ser/Ser). Recently, several researchers have reported this SNP, however, no functional analyses were conducted [24, 31, 32]. Sato et al. [33] identified that two novel LMX1B mutations located in homedomain can diminish transcriptional activity and affect DNA binding ability. Isojima et al. [7] reported that one novel mutation (R246Q) in exon4 can result in lower transcriptional activity. Moreover, results from the powerful tools RESCUE-ESE and ESRSearch of F-SNP revealed that this variant possibly located in exonic splicing enhancers may disrupt alternative splicing and reduce activity [34, 35]. Further functional research into effects of this SNP causing steroid resistance remains to be conducted.

The haplotype analysis among INS patients revealed that T-C haplotype carriers were in higher risk to developing steroid resistance. These LMX1B haplotypes were located in block 4 [36]. It was not acknowledged how these haplotypes may be functionally associated with the steroid responsiveness. Isojima et al. [7] suggested that LMX1B haplo-insufficiency can cause isolated glomerulopathy with irregular thickened GBM and deposition of Type III collagen. Therefore, haplo-insufficiency in LMX1B, which can result in some degree of podocyte dysfunction, may be an explanation for it. In conclusion, the LMX1B haplotype analysis confirmed that the haplotype combined by the rs34682917 and rs13295990 polymorphism was significantly associated with steroid responsiveness and may serve as a predictor for steroid resistance in INS patients.

Nevertheless, some limitations in this study have to be mentioned. The major limitation was the relatively small patients studied in a single center, especially patients with renal biopsy. Thus, it is necessary to investigate the association between LMX1B gene polymorphisms and the renal pathology, and to confirm these results in larger multi-centric studies. Then, we could not compare early and late responders since the exact time to remission was not known in some patients.

To conclude, our results demonstrate that genetic variations in the LMX1B gene are risk factors for increased steroid resistance and may serve as a predictor for initial steroid responsiveness in INS patients. Further studies are necessary to confirm a correlation in LMX1B mRNA and protein expression of these patients and to replicate this research among different ethnicities.

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Disclosure of conflict of interest

None.

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