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Mutation analysis of TRPC6 gene in nephrotic syndrome in children from a single center

Junhua Deng[1], Zengpo Huang[1], Sanju Yang[2], Yunguang Liu[1*]

[1]Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China
[2]Youjiang Medical University for Nationalities, Baise, China
[*]Corresponding author: Lyg9226@163.com

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Abstract: We analyzed the correlation between two single nucleotide polymorphism sites (rs12366144 and rs7931399) on TRPC6 gene and primary nephrotic syndrome (PNS). 205 cases of Guangxi Zhuang children were selected, of which 108 cases were in the PNS group and 97 cases were in the healthy control group who came to our hospital for physical examination in the same period. The rs12366144 and rs7931399 loci of the TRPC6 gene were genotyped using second-generation genetic testing technology, and their correlation with the development of PNS was analyzed. Logistic regression analysis was used for association analysis. Haplogroup construction was also performed for both loci using SHEsis software to analyze the association between both loci and PNS. The polymorphisms at rs12366144, rs7931399 loci of TRPC6 gene may not be significantly related to the development of primary nephrotic syndrome in Guangxi Zhuang children.

1. Introduction

Primary nephrotic syndrome (PNS) is one of the most common urologic disorders in children, often presenting with massive proteinuria and hypoproteinemia[1]. The pathogenesis of PNS has not yet been fully investigated. In recent years, the study of nephrotic syndrome and renal podocytes is also a research hotspot. Foot cell injury can cause proteinuria and glomerular basement membrane detachment. In addition to "pathological" podocytes and their detachment, podocyte injury also causes glomerulosclerosis[2]. To date, several gene mutations associated with podocyte injury have been identified, including NPHS2, NPHS1, WT1, TRPC6, PLCE1, ACTN4, etc[3-5]. Farmer et al. [6] knocked out the TRPC6 gene in mice in the laboratory, and found that upon loss of the TRPC6 gene, calpain-1 (calpain 1, which regulates the motility of the podocyte cytoskeleton) was activated. cytoskeleton movement) was activated, leading to focal segmental glomerulosclerosis. In this study, we used second-generation sequencing technology to detect the rs12366144 and rs7931399 loci on the TRPC6 gene in 108 patients with PNS and 97 healthy controls ,for understanding the mutation status, and calculated the genotypes and allele frequencies of these two loci, as well as the LD values and constructed haplotypes of the two loci, in order to explore the relationship between these two loci and In order to explore the relationship between these two loci and the development of primary nephrotic syndrome in Guangxi Zhuang children, it is of great significance to further reveal the pathogenesis of nephrotic syndrome.

2. Study data and methods

2.1 Clinical data

The clinical study subjects were 108 children with primary treatment of PNS who attended the pediatric department of the Affiliated Hospital of *Youjiang Medical University for Nationalities* from October 2018 ~ October 2022. There were 83 males and 25 females, aged (8.402 ±3.17) years.The diagnostic criteria of PNS and the effect of hormone therapy were in accordance with the Kidney Disease Treatment Guidelines of the United States (KDOQI)[7]. The control group was 97 healthy children, 65 males and 32 females, who were examined at the same period in our hospital. Age (9.2±3.04) years.The children in the PNS group and the healthy control group had three or more generations of grandparents and grandchildren, all of whom were of Zhuang ethnicity. There was no statistically significant difference between the age and gender of the two groups after statistical calculation (P>0.05). The families of the children were informed of the study and their consent was obtained, and all the children's families signed an informed consent form. The study was approved by the Ethics Committee of the Right River College of Ethnic Medicine.

2.2 Selection of SNP loci

Querying the NCBI database in the United States, a total of 52,595 single nucleotide polymorphism sites of TRPC6 gene were registered. The minimum allele frequency was set to 0.01 for Beijing, China, and the Chinese and English databases, including China Knowledge, Wanfang, Wipro, pubmed, web of science, etc. were used as reference. Finally, rs12366144 and rs7931399 were selected as the two single nucleotide polymorphism loci.

2.3 TRPC6 gene mutation and polymorphism detection

2.5 ml of peripheral venous blood was drawn from both groups, put into EDTA test tubes, and DNA was extracted with DNA extraction kit and stored in the refrigerator at minus 20 degrees C. The DNA was extracted by direct sequencing technology, and the TRPC6 gene was sequenced and typed. The TRPC6 gene was sequenced by direct sequencing technology and its polymorphisms were typed. Primers were synthesized using the standard human genome as a template. PCR amplification was used to introduce specific tag sequences to the end to obtain the required FastTarget sequencing gene library fragments, and the gene fragments of the library were validated and accurately quantified after high-throughput sequencing to obtain the required FastQ data.

2.4 Statistical methods

Data were processed using SPSS24. 0 software, plink1.9 software and SHEsis software, and the Hardy-Weinberg balance test was performed on the healthy control group to assess whether it was representative of the group. The t-test was performed for measurement data and the χ^2 test (or Fisher's test) was performed for count data. ORs and 95% confidence intervals were calculated for genetic polymorphic loci and risk of disease using plink software. The χ^2 test (or Fisher test) was used for differences in haplotype frequency of haploid constructs for single nucleotide polymorphism loci using SHEsis software.

3. Results

3.1 Hardy-Weinberg balanced test

Hardy-Weinberg equilibrium test was performed on two points (rs12366144 and rs7931399), in which rs12366144 χ^2=0.4216, p=0.5161, rs7931399 χ^2=0.1794, p=0.6718. P value of both points is greater than 0.05, and this sample is representative of the group.

3.2 Polymorphism analysis of rs12366144, rs7931399 loci

The difference between the PNS group with rs12366144, rs7931399 loci of TRPC6 gene and the healthy control group in the co-dominant, dominant, recessive model analysis of genotype distribution and allele frequency comparison results were not statistically significant (P > 0.05), and there was no significant difference between the two groups (Table 1). Logistic regression analysis under the dominant, superimposed model, the two points were not associated with the development of PNS in Guangxi Zhuang children (Table 2).

3.3 Chain imbalance analysis

LD chain disequilibrium analysis was performed on rs12366144, rs7931399, and found that the haplotype chain disequilibrium of the two points was not strong and significant (D'= 0.864 r2= 0.487) (Fig. 1, Fig. 2).

3.4 Haplotype analysis

Haplotypes were constructed using SHEsis software for the PNS group and healthy control group, and it was found that three haplotypes AA,GA,GC existed in both groups. The frequency analysis of these three haplotypes between the PNS group and the healthy control group showed no statistical significance (P>0.05). It showed that these three haplotypes were not significantly associated with the development of PNS in Guangxi Zhuang children (Table 3).

Table 1: Frequency results of the distribution of rs12366144 and rs7931399 genotypes and allele frequencies of the TRPC6 gene in children in the PNS group and healthy control group

SNP	MODEL	GENOTYPE	CASE	CONTROL	χ^2	P
rs12366144	Codominant	AA	97	85	1.337	0.576
		AG	10	12		
		GG	1	0		
	Dominant	AA	97	85	0.245	0.621
		AG+GG	11	12		
	Recessive	GG	1	0		0.527
		AA+AG	107	97		
	Allele	A	204	182	0.074	0.786
		G	12	12		
rs7931399	Codominant	AA	100	89	0.05	0.823
		AC	8	8		
		CC	0	0		
	Dominant	AA	100	89	0.05	0.823
		AC+CC	8	8		
	Recessive	CC	0	0		
		AA+AC	108	97		
	Allele	A	208	186	0.048	0.826
		C	8	8		

Table 2: Dominance of TRPC6 genes rs12366144 and rs7931399 in children of PNS group and healthy control group, results of logistic regression analysis of superimposed model

SNP	MODEL	GENOTYPE	CASE	CONTROL	OR	L95	U95	P
rs12366144	Dominant	AA	97	85	0.8033	0.3371	1.914	0.621
		AG+GG	11	12				
	Additive				0.8948	0.3963	2.02	0.789
rs7931399	Dominant	AA	100	89	0.89	0.3207	2.47	0.8229
		AC+CC	8	8				
	Additive				0.89	0.3207	2.47	0.8229

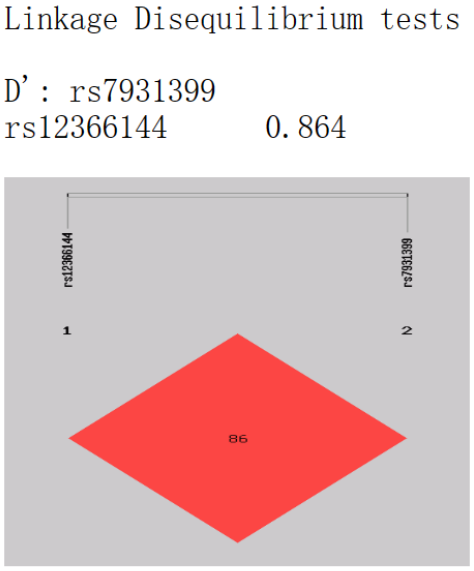

Figure 1: D' values of LD linkage disequilibrium analysis for rs12366144, rs7931399.

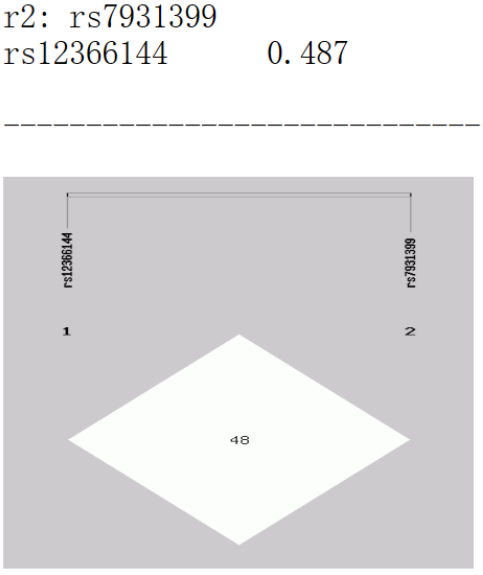

Figure 2: rs12366144, rs7931399 LD linkage disequilibrium analysis of r2 values for both sites

Table 3: Results of gene haplotype construction analysis of TRPC6 gene rs12366144 and rs7931399 in children of PNS group and healthy control group

		Case(freq)	Control(freq)	Chi2	Fisher's p	Pearson's p	OR	[95%CI]
A	A*	204.00(0.944)	179.93(0.927)	0.089	0.765351	0.765343	1.134	[0.497~2.587]
A	C	0.00(0.000)	2.07(0.011)	-	-	-	-	
G	A*	4.00(0.019)	6.07(0.031)	0.725	0.39452	0.394479	0.578	[0.161~2.073]
G	C*	8.00(0.037)	5.93(0.031)	0.116	0.733272	0.733267	1.206	[0.410~3.554]

4. Discussion

Focal glomerulosclerosis caused by mutations in the TRPC6 gene has been a hot research topic over the years[8,9].Since 2005, when winn et al[10] first cloned the TRPC6 gene that causes FSGS from patients with familial focal segmental glomerulosclerosis (FSGS), the TRPC6 gene and the TRPC6 cation channel protein have been continuously research [11].There are interactions between TRPC6 cation channel proteins and the cleavage septum molecules Nephrin and Podocin, which are jointly involved in physiological functions such as intercellular signaling between podocytes, cell polarization, and stabilization of the cytoskeleton [10].In 2018, scholars Chen Lei et al [12] observed by cryo-electron microscopy that TRPC6 consists of amino acids that are folded into two long helices, namely CH1 and CH2.CH1 runs from the periphery to the center of the channel and connects to CH2 through a 90 ° turn.And it was seen that TRPC6 protein is a 6-transmembrane protein consisting of 931 amino acids, with a small pore region between the 5th and 6th transmembrane proteins, which forms the cation channel.Wang Q et al [13] stated that in damaged podocytes, TRPC6 protein regulates cation channels through the Rho/ROCK dependent pathway regulates foot cell actin stress fiber formation and focal adhesions. New studies demonstrated that nephrin and TRPC6 proteins have direct or indirect interactions in podocytes[14].

Scholars knocked out the TRPC6 gene in the Akita mouse model and found that mice had reduced renal tubular injury but increased mesangial dilation. The Akita mouse overexpression model leads to FSGS[15]. In the same year, Kim et al [16] gene edited to delete a region of the TRPC6 exon in rats and found that renal tubular interstitial fibrosis and loss of pedunculated protrusions with thickening of the basement membrane were reduced in the experimental group as compared to the healthy group.Feng Y et al [17] activated the TRPC6 gene using angiotensin II in rats and caused it to be highly expressed, which led to pedunculated cell damage in the rats and the production of large amounts of proteinuria. This seems to indicate that complete deletion of the TRPC6 gene does not protect the podocytes, and that its high, low or no expression causes damage to glomerular podocytes.

Mutations in the TRPC6 gene play an important role in primary nephrotic syndrome in human children.Weizhen Tan et al [18] American scholars used microfluidic technique of high-throughput PCR to analyze mutations in 24 single genes in children with SRNS from 72 families, and as a result, mutations in the TRPC6 gene were detected (c.2683C>T, p.R895C, heterozygous), which confirms that the TRPC6 gene can cause FSGS. In addition, according to the report, Japanese researchers found for the first time that a boy aged one year and one month suffered from diffuse mesenchymal sclerosis (DMS) due to a heterozygous missense mutation in the TRPC6 gene (NM_004621.5: c.523C > T: p.Arg175Trp)[19], which suggests that the TRPC6 Joshi B B, Koringa PG et al [8] Indian scholars conformationally analyzed and verified that both mutations in TRPC6 (N157T, rs35857503; A404V, rs36111323) are important influences in causing FSGS in almost all four instruments. Wang F,ZhangY et al[20] Chinese scholars performed gene sequencing on 110 children with SRNS from five research centers in China and detected a de novo missense mutation in TRPC6 (c.523C > T, p.Arg175Trp) in one case of infantile nephrotic syndrome, suggesting that mutations in the TRPC6 gene would lead to infantile nephrotic syndrome This suggests that TRPC6 mutations will lead to the

development of nephrotic syndrome in infants and children. Studies have shown that TRPC6 gene mutation can not only lead to primary nephrotic syndrome, but also altered TRPC6 protein channels can lead to prostate cancer[21], gastric cancer [22], breast cancer [23] and other diseases. There are also some data suggesting that when TRPC6 channels are dysregulated, it can cause increased foot cell damage and may even lead to diabetic nephropathy [224,25].

The genotype distribution and allele frequencies of rs12366144 and rs7931399 were not statistically different between children with PNS and the normal population. This suggests that the polymorphisms at these two loci may not be related to the development of PNS. LD linkage disequilibrium analysis of rs12366144, rs7931399 showed that the haploid linkage disequilibrium of the two loci was not strong and significant. The haplotypes were constructed using SHEsis software for the PNS group and the healthy control group, and it was found that there were three haplotypes, AA, GA and GC in both groups, and the frequency analysis of these haplotypes between the PNS group and the healthy control group showed no statistical significance ($P > 0.05$). It showed that these three haplotypes were not significantly associated with the development of PNS in Guangxi Zhuang children. Currently, scholars have various opinions about TRPC6 gene mutation causing kidney disease. In the past, some scholars have argued that， mutations in the TRPC6 gene lead increasing calcium inward flow through the channel, causing podocyte injury[26]. However, it has also been found that by inducing TRPC6 overexpression with angiotensin II, it can also cause foot cell injury[27]. It has also been suggested that physical interactions between TRPC6 protein and calpain 1 and 2 in podocytes are important for cell motility and detachment, and a scaffolding role for TRPC6 protein in disease has been demonstrated[6]. For the study of TRPC6 gene mutation sites, the choice of detecting promoters and exons is more common, and the choice of introns, terminators is less common. Some scholars have examined the -254C>G site of the promoter of TRPC6 and found that the -254C>G SNP enhances nuclear factor-κB-mediated promoter activity and stimulates TRPC6 expression and leads to idiopathic pulmonary arterial hypertension (IPAH) [28]. It has also been shown that reduced gene transcription of NF-κB in renal cells can lead to decreased TRPC6 expression[29]. It was found[30] that -254C>G SNP enhanced the transcription of the TRPC6 promoter in vitro and was associated with increased TRPC6 expression in renal tissues of SRNS patients. Some scholars found that children with IMN at the rs12366144 locus in exon 6 of the TRPC6 gene differed from healthy controls ($P=0.02$), and suggested that this locus is associated with the development of IMN [31]. The loci selected in this study were rs12366144, rs7931399, which are located in the exon and terminator of TRPC6 gene, respectively. Checking the domestic and international literature, research reports on the rs12366144, rs7931399 polymorphic locus of TRPC6 gene associated with PNS are rare. Some scholars isolated genomic DNA from blood samples of 101 iMN patients and 292 controls.Then direct sequencing of the entire TRPC6 gene revealed no differences in genotypes or allele frequencies at the rs12366144 locus, and suggested that most of the currently identified SNPs are mostly intronic or synonymous mutations, greatly reducing the possibility of any functional association[31]. The rs7931399 locus is located in the terminator of TRPC6 and does not participate in TRPC6 protein coding, and no studies on this locus have been reported at home or abroad. In the present study, by testing the TRPC6 gene in children with PNS, we found that the genotype or allele frequency of this locus in children with PNS did not differ from that of healthy controls, which completes the research gap of the rs7931399 locus. Meanwhile, the results of this study on TRPC6 in children with PNS may indicate that these two loci are not involved in the development of primary nephrotic syndrome.

In summary, this study reported for the first time that polymorphisms and haplotypes at the rs12366144 and rs7931399 loci of the TRPC6 gene were associated with PNS in Guangxi Zhuang children. And it was found that these two loci were not involved in the occurrence of primary nephrotic syndrome. Due to the small sample size and the fact that only two loci on the TRPC6 gene

were selected for sequencing and analysis, it was not possible to determine the association of other loci of the gene with primary nephrotic syndrome. Therefore, the correlation between TRPC6 gene and PNS in children still needs to be expanded as well as further in-depth studies.

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