NPHS2 variation in children with late steroid-resistant nephrotic syndrome

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Abstract: Many children with late steroid resistent nephrotic syndrome (late SRNS) are prone to a complicated clinical and therapeutic course.The etiopathogenesis and the long-term prognosis of late SRNS remains obscure. Considering the similar steroid resistance between late and initial SRNS, analysis of NPHS2 variation was performed in 35 sporadic Chinese children with late SRNS and 30 controls in present study to investigate the possible role of NPHS2 gene in late SRNS. The variation analysis revealed 3 polymorphisms (288C>T heterozygous in exon 2, 954T>C heterozygous and homozygous, 1038A>G heterozygous in exon 8) in 11 out of 35 patients studied, but there was no significant difference in the genotypic and allelic frequencies of these polymorphisms between patients and controls. The hypothesis that late SRNS may be associated with NPHS2 gene variations was not confirmed in this studied..

[Ren Q ,YU SY. **NPHS2 variation in children with late steroid-resistant nephrotic syndrome.]** New York Science Journal 2011;4(9):30-33]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork

Keywords: NPHS2; nephrotic syndrome; mutation; SRNS

1. Introduction

Nephrotic syndrome (NS) is a common disease in pediatric nephrology and represents the asscociation of proteinuria, hypoalbuminemia, edema and hyperlipidemia. In Europe and America 80% of NS children are sensitive to typical steroid treatment (steroid sensitive nephrotic syndrome, SSNS) with eusemia, and approximately 25% NS children are primarily resistant to the treatment (steroid resistant nephrotic syndrome, SRNS) with a possibly gradual progression to end-stage renal disease (ESRD)[1]. Of the children with SSNS, about 4~10% will display a late steroid resistance (late SRNS)thereafter [2~3]. Many of them are prone to a complicated clinical and therapeutic course, however the etiopathogenesis of late SRNS remains unclear.

There is now growing edvidence that genetic variation is one of the causes of SRNS. Mutations of NPHS2 gene have been reported in 26-38% of familial SRNS and 10.5~23% of sporadic cases in children in a large European survey[4~6], but have not been well-characterized in late-onset SRNS. Considering the similar steroid resistance between late and initial SRNS, analysis of NPHS2 variation was performed in 35 sporadic Chinese children with late SRNS to investigate the possible role of NPHS2 gene on late SRNS.

Subjects and methods

2. Material and Methods

Patients and control

Inclusion criteria were: (1) identified with a clinical diagnosis of SSNS; (2) responded to the initial standard steroid treatment (a daily corticosteroid (prednisone or prednisolone) at a 2 mg/kg/day (maximum 60mg/day) for 4 weeks and were theh switched to alternate day therapy) and then developed a steroid resistance later in the course of treatment; (3) from unrelated families. 35 eligible patients (19 male and 16 female) were followed by two regional centers for pediatric diseases in China for 4~76 months (mean 3.2years).The median onset age of the disease was 4.8 years old (8months~14years). Patients with congenital NS (NS occurring before 3 months of age) and secondary NS were excluded. Controls included 30 healthy children with age and ethnic background matched and without personal or family history of a kidney disease. The study was approved by The Ethic Committee of the two regional centers for pediatric diseases. Written informed consent was obtained from each subject or their parents.

The definitions and criteria were: NS was defined as massive proteinuria (≥40mg/m2/h), hypoalbuminemia (serum albumin＜25g/L）edema and hyperlipidemia. A partial response was defined as disappearance of edema, an increase in the serum albumin concentration to >35 g/L, and the persistence of proteinuria of ＞4mg/m2/h.. Steroid-resistant was defined as the persistence of proteinuria after initial daily corticosteroid (prednisone) treatment at 2.0mg/kg/d for at lest 6 weeks. Patients were categarized as being steroid-sensitive if at least a partial response to steroids was observed. Late SRNS was defined as no response of a relapsing proteinuria to steroids in a patient who had initially reponded to steroids[7].

Variation analysis

Genomic DNA was directly isolated from peripheral blood samples, and subjected in touchdown PCR amplification in a total volume of 25ul, which contained 50ng templates, 5 pmol of each primer, 2×Taq platinum PCR masterMix 12.5ul (Qiagen, Germany) and ddH2O 9.5ul. DNA was denatured at 94℃ for 5 min; Then the annealing temperature was lowered 1℃ every 2 cycles from 64 to 59℃, followed by the annealing temperature of 58℃ for 26 cycles; At last the extension temperature stayed constantly at 72℃ for 7 min. The sequences of the oligonucleotide primers were given in Table 1.

Variation analysis was performed with directly sequencing of one strand of each exon with an automated sequencer (ABI PRISM 3735 , America) after cycle sequencing reactions and analyzed using Chromas 2.23 software. When the results were in doubts, the complementary strand was also sequenced. All variants were confirmed with sequencing of the complementary strand.

Statistical analysis

All the data were presented as mean±standard deviation or as percentage. Chi-squared test were used to analysis the difference of genotypic and allelic frequencies between patients and controls by SPSS software. A P-value of less than 0.05 was viewed as statistically significant.

Table 1 Sequences of primers used for touchdown

Exon	Forward primer	Reverse primer
1	AGCGACTCCACAGGGACTGC	CTGACGCCCCTTAGTTACCA
2	AGAATTGGACCAACAGATGC	AAGTGAGAATGGGCATGGTG
3	GCCCCCGCGGTCTTATGCCAAGGCCTTTTGAAGAC	GGGTTGAAGAAATTGGCAAGTCAGG
4	AAGGTGAAACCCAAACAGC	CGGTAGGTAGACCATGGAAA
5	AGGATTTACCACAGGATTAAGTTGTGCA	TAGCTATGAGCTCCCAAAGGGATGG
6	TATTTATAAATAAGGCACTGTGAAGTTAAATACAAC	CCCCCGCCCGCCAGAATATTTTCCTTTATCATACAG
7	GAGGCTTGCAAGTCTGTGTGAAAGC	AGGAAGCAAAGGGGAAATGTTCTCC
8	ATGCTCAGTGCTTGTCTGCT	TCACATTATGCCCCATCCTT

3. Results

During the follow-up period, 26 patients were treated with cyclophosphamide and prednisone ; 4 patients achieved complete remission with prolonged steroid therapy. A large number (19 patients) again became steroid sensitive. 25 patients maintained stable renal function: 16 remained in remission (10 received cyclophosphamide, 3 received cyclosporine A and 3 received both drugs subsequently), 6 showed recurrence of proteinuria and 3 experienced patial response. 4 patients with abnormal renal function did not show response to intravenous cyclophosphamide, and one progressed to ESRD followed dislysis (no.2). One was dead from serious infections. Renal histologic examination, which was available for 12 patients, revealed minimal change lesions (MC) in 6 patients, focal segmental glomerulosclerosis (FSGS) in 3 patients(no.2 and no.4), mesangioproliferative glomerulonephritis in 1 patient and 2 patients showed a transition from MC to FSGS in repeat biopsies.

No mutation was found in all patients and controls. Three sequence variations as known single nucleotide polymorphism (SNP) (288C>T heterozygous in exon 2, 954T>C heterozygous, 954T>C homozygous and 1038A>G heterozygous in exon 8) were observed, as summarized in Fig1. To exam the significance of the variations, the same exons were also sequenced from 25 control subjects, and the results were compared with published data and database(dbSNP, SNPper). These SNPs were found in 35 patients and 30 controls respectively, and none of them caused an amino acid substitution. There is no significant difference in clinical characteristics between patients with and without SNPs. The clinical and NPHS2 variations of our patients carrying NPHS2 gene polymorphisms are summaried in Table 2.

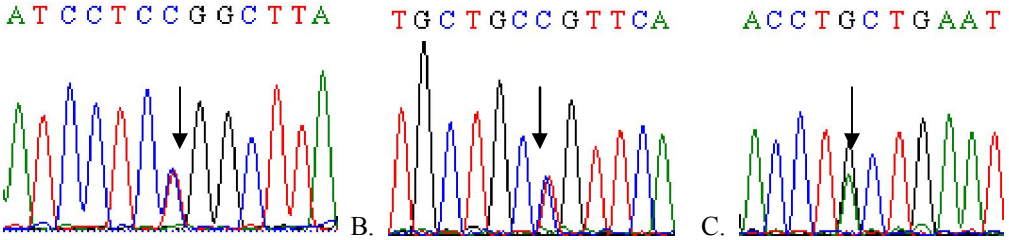

Fig.1 Direct sequencing Exon in NPHS2 The arrows indicate:Fig.A—NPHS2 288C>T heterozygous variation in exon 2; Fig.B—954T>C heterozygous in exon 8; Fig.C—1038A>G heterozygous in exon 8.

Table 2. The clinical and NPHS2 variations of 11 patients with SNPs

Patient	Gender	Age at onset of INS(years)	Period of observation (years)	Time until late resistance from onset of INS (months)	Relapses until late resistance	Cytostatic drugs	Variation
1	F	10.2	3.0	3.0	12	CsA	288C>T(h) 954T>C(h) 1038A>G(h)
2	M	6.1	2.6	5.3	10	CsA/ Cph	288C>T(h) 954T>C(h) 1038A>G(h)
3	M	2.3	0.7	13	2	Cph	954T>C(h)
4	F	4.5	2.1	6.2	14	CsA/ Cph/ CsA	288C>T(h) 954T>C(h) 1038A>G(h)
5	F	7.6	3.3	4.2	5	Cph	954T>C(H)
6	M	0.8	2.7	7.9	6	CsA	954T>C(h) 288C>T(h)
7	M	3.3	4.0	4.2	2	CsA	954T>C(H)
8	M	1.6	2.7	10.0	4	CsA	954T>C(h)
9	F	5.4	4.2	4.5	7	CsA	954T>C(h) 1038A>G(h)
10	M	1.4	1.2	6.2	4	Cph	954T>C(h)
11	M	2.9	0.5	4.0	3	Cph	954T>C(h)

F famale, M male, PNS primary nephrotic syndrome, CsA cyclosporin A, Cph cyclophosphamide, h heterozygous, H homozygous.

4. Discussions

Late SRNS represents a clinical heterogeneous group that is initially sensitive to standard steroid treatment but develops a late steroid resistance. The patients with late SRNS show a steroid-sensitive NS or frequently relapsing NS in the first few weeks and then develop a typical clinical course of SRNS. Most of them display a persistent proteinuria and have to be treated with other therapy protocol, i.e. immunosuppressive therapy. Recessive mutations of NPHS2 (podocin) are associated with the disruption of the slit diaphragm and the development of SRNS. The causal role of NPHS2 variation in initial SRNS is well established now. And the FSGS recurrence risk in a kidney transplant is significantly reduced in children bearing NPHS2 mutations relative to children without a mutation (8% vs 35%)[8]. To date, more than 50 NPHS2 mutations have been reported in published studies. The variations in the frequency and the type of NPHS2 mutations among different populations partially explain the inter-ethnic difference in the prevalence as well as the outcome of SRNS. Large-scale mutation studies of SRNS of various ethnic origins revealed that the NPHS2 mutations appear to be very uncommon in Far East Asian Countries (China, Japan and Korea)[9-11]. Conversely, the incidence of NPHS2 mutations was 25% in cases of sporadic SRNS in a Egyptian study[12], 4% in a Turkish study[13], and 10.5~23% in Euopean studies[4~6]. To our knowledge, there is few study on correlation between NPHS2 variations and late SRNS in Chinese children. In the present study, three SNPs (288C>T, 954T>C and 1038A>G) were observed in exon 2 and exon 8 of NPHS2 gene in 35 patients and 25 controls, which have been also reported in Chinese children with SRNS[10,14]. Although the 3 patients (2 with FSGS) carrying compound heterzygous variants (288C>T, 954T>C and 1038A>G) were all suffered more relapses until late resistance than others, the differnces were statistically not significant. Weber et al.[4] reported that R138Q appeared to be associated with early onset SRNS (12±3 months in 15 patients), whereas V180M and R238S were associated with late-onset SRNS (129±12 months in 7 patients). Schwaderer et al.[2] reported that uncommon variant of NPHS2 gene (IVS3+10insA) in late-onset patients which had no major influence on splicing mechanisms. However, none of the late SRNS patients with those variations, including R229Q which was discussed to play a role as disease modifier for glomerular disorders[15], was observed in this study, similarly to the data of Cho et al[11].

On the other hand, we reviewed the clinical pictures of late SRNS. The mean age at onset was 5.1 years, which was similar to the data of Kim et al. and Gulati et al.[3,16]. Despite such similarity of clinical features, we found a substantial difference in the time interval from disease onset to late resistance development. The intervals of older patients with more relapses were shorter than younger ones, which suggests that age at onset was a highly siginificant factor, and older children may have a higher tendency to develop late SRNS. In our patient group, 26 patients received cyclophosphamide and steroid therapy, whereas cyclosporine was applied in 8 patient who could not go into remission with the therapeutic alliance. 16 patients achieved complete remission followed therapy and only one patient developed

ESRD. Data from the present and precious studies showed that complete remission was more likely to occur in patients with late than with initial resistance(22.2%)[17]. So a first treatment attempt with cytostatic drugs might be justifiable. Renal biopsies showed MCD was the commonest pathology feature in late-onset SRNS. 5 patients with MCD experienced steroid sensitive relapse after becoming steroid resisitant. 2 patients with FSGS did not consent to cyclotatic drugs. Hence we suppose that there be a possible association between histology and response to CsA in late resistance as Gulati et al[16]. observed in his study. 2 patients showed MCD in early biopsy but FSGS in subsequent biopsy. Although complete remission was achieved in a higher proportion of patients with MCD compared with FSGS and MC, the differencs were not significant. And we can not exclude a bias resulted from the transition of histological pathologies in this study.

In conclusion, 3 SNPs in NPHS2 gene were identified in 35 Chinese children with late SRNS, though the results did not support the possible role of NPHS2 gene in susceptibility to late SRNS in children. Considering the fact that only sporadic late-onset SRNS children were included in the present study, it will be necessary to obtain future mutational analysis of both familial and sporadic late-onset SRNS for safer statement. With respect to the clinical course, late SRNS appears to resemble SSNS, and the majority of them have a favorable outcome with cytostatic drugs. Furthermore, other genes commonly involved in the pathogenesis of SRNS, CD2AP and TRPC6 for instance, should be considered in children with late-onset SRNS. And other endogenous or exogenous factors that act as modulators of the general immune response or that promot revesible podocyte damage remain to be identified.

Acknowledgements:
We thank patients and their parents for permission to publish the report.

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8/22/2011