IJKD KIDNEY DISEASES

Cytokine Gene Polymorphism in Children With Idiopathic Nephrotic Syndrome

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Original Paper

Introduction. Idiopathic nephrotic syndrome (INS) is a glomerular disease with completely unclear pathogenesis and different responses to steroid therapy. This study aimed to investigate the role of cytokine genes promoter polymorphisms in steroid therapy responses.

Materials and Methods. One hundred children with INS and 30 healthy controls were studied. Genotyping of TNF-α-G308A single nucleotide polymorphism was done using polymerase chain reaction-restriction fragment length polymorphism method, while of IL-6-G174C single nucleotide polymorphism was done using real-time polymerase chain reaction.

Results. The IL-6-G174C exhibited a significantly different genotype distribution among the children with INS compared with the controls (GG versus CC, $P = .02$; GG versus GC, $P = .003$; odds ratio [OR], 5.83; 95% confidence interval [CI], 1.64 to 20.70; as well as alleles distribution of G versus C, $P < .001$; OR, 7.57; 95% CI, 2.28 to 25.17). With regard to TNF-α-G308A genotype, there was no significant difference in genotype distribution of the children with INS compared with the controls, but a significant difference was observed at the alleles level. Comparing the steroid-resistant group with the steroid-sensitive group, significant association was found at genotypic level in case of IL-6-G174C (GG versus CC, $P = .03$; OR, 5.52; 95% CI, 1.39 to 21.89), but no association was found regarding GG versus GC. At the allelic level of IL-6-G174C, there was no significant association either.

Conclusions. IL-6-G174C and TNFα-G308A polymorphisms may affect susceptibility to idiopathic nephrotic syndrome and might affect steroid response in INS patients.

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INTRODUCTION

Nephrotic syndrome (NS) is a glomerular disease that is characterized by the presence of proteinuria (urinary protein excretion, > 3 g/d), hypoalbuminemia, hypercholesterolemia, generalized edema, and a relapse or remission course. Children are more commonly affected and its cause may be primary or secondary.[1] Idiopathic NS (INS) can be diagnosed by determination of urine protein-creatinine ratio in a random urine sample or measurement of urinary protein in a 24-hour urine collection.[2] Childhood NS may be idiopathic (90%), secondary (10%), and congenital (< 1%). Idiopathic NS has 3 histological variants of minimal

change NS, focal segmental glomerulosclerosis, and membranous nephropathy.[3,4]

Changes of capillary endothelial cells, the glomerular basement membrane, or podocytes are the causes of proteinuria. In primary and secondary glomerular disease, the mechanism of damage to these structures is unknown, but evidence suggests that T cells may upregulate a circulating permeability factor or downregulate an inhibitor of permeability factor in response to unidentified immunogens and cytokines.[5] Minimal change disease is often associated with atopy and allergy, which are caused by helper T2 immunologic responses.[6,7] Helper T2 cells produce interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-13, which provide optimal help for antibody production and promote both mast cell growth and eosinophil activation causing humoral response.[8]

The increased serum immunoglobulin E level and the preservation of immunoglobulin G4 observed in minimal change disease are also characteristic of a helper T2 response.[9] Cytokines play a critical role as mediators of inflammation and are considered as prime candidates for mediating INS progression.[9] We investigated the role of IL-6-G174C and TNF-α-G308A. Cytokines genes promoter polymorphisms in INS children and their relation to the response to steroid therapy in these children.

MATERIALS AND METHODS

Participants

This study was carried out at the Medical Biochemistry and Pediatrics Departments, Faculty of Medicine, Menoufia University. The study protocol was approved by the ethics committee of Faculty of Medicine, Menoufia University. A written informed consent was obtained from all parents of the included children. One hundred children with NS were enrolled in the study and 30 age- and sex-matched healthy children served as controls.

Nephrotic syndrome was diagnosed with the presence of proteinuria of 40 mg/m^2/h or spot urine protein-creatinine ratio of 2 in the first-morning urine sample, hypoalbumenemia (serum albumin, < 2.5 g/dL), hyperlipidemia (serum cholesterol, > 200 mg/dL), and edema. The remission of NS was defined by urinary protein excretion less than 4 mg/m^2/h or urine dipstick nil or trace for 3 consecutive days. The relapse was defined as proteinuria of 40 mg/m^2/h or more or 2+ dipstick or more for 3 consecutive days. Frequent relapses were defined by 2 or more relapses within 6 months of initial response or 4 or more relapses within any 12 months period. The steroid dependence was defined by 2 consecutive relapses occurring during the period of steroid taper or within 14 days of its cessation. Kidney biopsies were carried out in all steroid-resistant cases.

The children with NS were subgrouped according to the response to steroid therapy into 2 groups of steroid-responsive group (infrequent relapses, frequent relapses, and steroid dependent) and steroid-resistant group. The genotyping was performed for all the included children. A thorough history and full clinical examinations were obtained.

Methods

Blood sampling. Seven-milliliter venous blood samples were collected from every participant and divided into 3 tubes. One milliliter of venous blood was put in ethylenediaminetetraacetic acid-containing tube for hemoglobin measurement; 4 mL of venous blood were put in ethylenediaminetetraacetic acid-containing tube for DNA analysis; and 2 mL were put in plain tube where sera were used for measurement of blood urea nitrogen, creatinine, total cholesterol, and total protein. Analysis was done on a Beckman Coulter AU480 autoanalyzer.

DNA analysis. Genomic DNA was extracted from ethylenediaminetetraacetic acid-anticoagulated peripheral blood (QIAamp DNA Blood Mini Kit, Qiagen).

Genotyping of tumor necrosis factor (*TNF)-α-308G/A* single nucleotide polymorphism was carried out by polymerase chain reaction (PCR)-restriction fragment length polymorphism method. A fragment containing the polymorphic site was amplified using the primers 5′-AGGCAATAGGTTTTGAGGGCCAT-3′(forward) and 5′-TCCTCCCTGCTCCGATTCCG-3′ (reverse). In a total volume of 25 µL, the reaction mixture contained 12.5 µL Dream Tag green PCR master mix (Thermo Scientific), 1 µL of each primer, 10 µL of genomic DNA, and 0.5 µL of DNase and RNase free water. Polymerase chain reaction conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 60 seconds, and 72°C for 60 seconds, and a terminal elongation step at 72°C for 7 minutes. Ten microliters of the PCR product (107

bp) were restricted by 1 µL of fast digest restriction enzyme Nco1 (Thermo Scientific) at 37°C for 10 minutes with 107-bp fragment corresponded to A allele (restriction site absent) and a set of 87-bp and 20-bp fragments corresponded to G allele (restriction site present) after separation in 2% agarose gel containing ethidium bromide as shown in Figure 1.[10-13]

Genotyping of *IL-6-174G/C* single nucleotide polymorphism was carried out by real-time PCR (RT-PCR) by allele discrimination using TaqMan single nucleotide polymorphism genotyping on Applied Biosystems 7500 RT-PCR System (Applied Biosystems, USA). During TaqMan single nucleotide polymorphism genotyping assay experiment, DNA polymerase from the TaqMan Universal PCR Master Mix amplifies target DNA using sequence-specific primers supplied with the kit. TaqMan two specific fluorescence-labeled probes provide a fluorescence signal for allelic discrimination using the dyes 6-carboxyfluorescein FAM (excitation, 494 nm) and VIC (excitation, 538 nm) which are easily differentiated in RT-PCR system. The reaction mixture (25 µL total volume per single well reaction) containing 12.5 µl of TaqMan Universal Master Mix 2X (Applied Biosystems, USA), 1.25 µL of TaqMan Assay 20X (which contains primers [5′-TAGCCTCAATGACGACCTAAGCT-3′; 5′-GGGCTGATTGGAAACCTTATTAAG-3′] and TaqMan Probes dye mix [VIC: TGTCTTGCCATGCTA, FAM: TGTCTTGCGATGCTA], 10.25 µL of RNase- and DNase-free water and 1 µL of DNA template). The cycling conditions included a 10 minutes of predenaturation (AmpliTaq Gold DNA polymerase activation), followed by 45 cycles with a fast denaturation at 95°C for 15 seconds, annealing of the TaqMan MGB probes to its complementary sequence and extension of the primers by AmpliTaq Gold DNA polymerase for 1 minute at 60°C.

After assay completion, the 96-well PCR plates were read on an Applied Biosystems 7500 RT-PCR system with endpoint analysis mode of the SDS v1.3.1, which uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample then convert allele calls to genotypes (VIC dye detects the G allele sequence and FAM dye detects the C allele sequence) as shown in Figures 2, 3, and 4.[14]

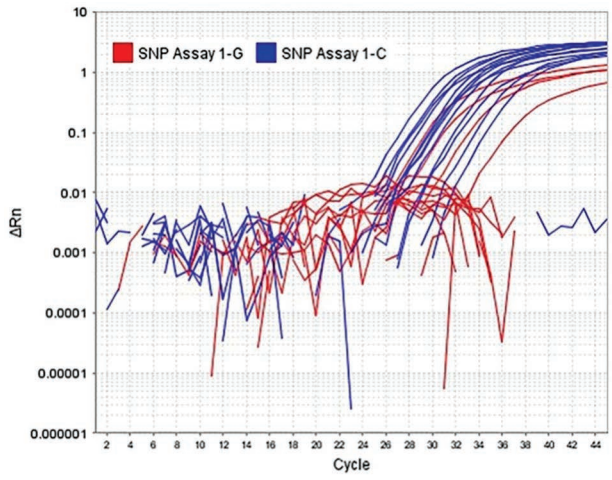

Figure 2. Amplification plot by real-time polymerase chain reaction of IL-6-174 G>C single nucleotide polymorphism (SNP). The amplification plot (ΔRnvs cycle) of IL-6-174 G>C SNP TaqMan assay genotyping linear view displays ΔRn values, which is the magnitude of the fluorescence signal generated during the polymerase chain reaction at each time point, on the Y axis. The increase in fluorescence emission is a consequence of target amplification during polymerase chain reaction. The Applied Biosystems 7500, software version 2.0.1, calculated the change in fluorescence emission as ΔRn values and constructed the amplification plot in which ΔRn values are plotted versus cycle numbers.

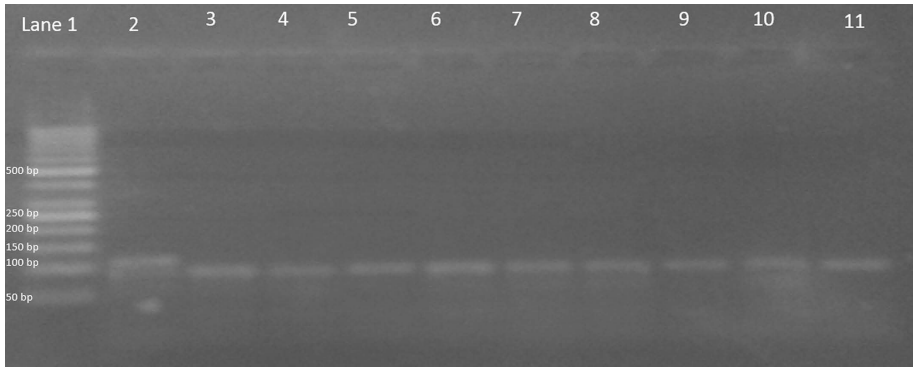

Figure 1. Results of restriction of TNF-α-G308A polymerase chain reaction product. Lane 1 is 50-bp DNA ladder; lanes 2 shows the AG genotype (107 bp, 87 bp, and 20 bp are not shown); and lanes 3, to 11 show the GG genotype (87 bp and 20 bp are not shown).

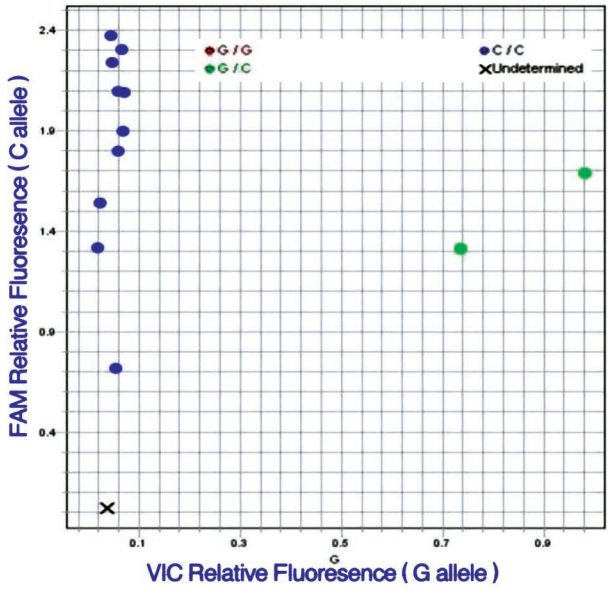

Figure 3. Allelic discrimination plot for IL-6: allelic discrimination plot of TaqMan genotyping assay for IL-6-174 G>C single nucleotide polymorphism with VIC dye fluorescence at X axis and FAM dye fluorescence at Y axis. The blue dots indicate individuals homozygous for the mutation (CC genotype) and the green dots indicate individuals heterozygous for the mutation (GC genotype). This scatter plot was obtained from TaqMan genotyping using the Applied Biosystems 7500, software version 2.0.1.

Statistical Analysis

The SPSS software (Statistical Package for the Social Sciences, version 14.0, SPSS Inc, Chicago, IL, USA) was used for analyses. The chi-square test and the Fisher exact test were used when comparing qualitative data. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the risk conferred by a particular allele and genotype. The Student *t* test was used to test the difference between normally distributed quantitative data among the studied groups. The Mann Whitney U test was used to test the difference between not normally distributed quantitative data among the studied groups. All tests were two-tailed, and statistical significance was assumed at a *P* value less than .05.

RESULTS

One hundred children with NS (63% boys and 37% girls) were enrolled in the study and 30 age- and sex-matched healthy children served as controls. The mean age was 7.51 ± 3.12 years for children with NS and 8.47 ± 3.80 years for the controls. All patients were presented with generalized edema, 32% of the patients were presented with hypertension, and 23% of them had hematuria.

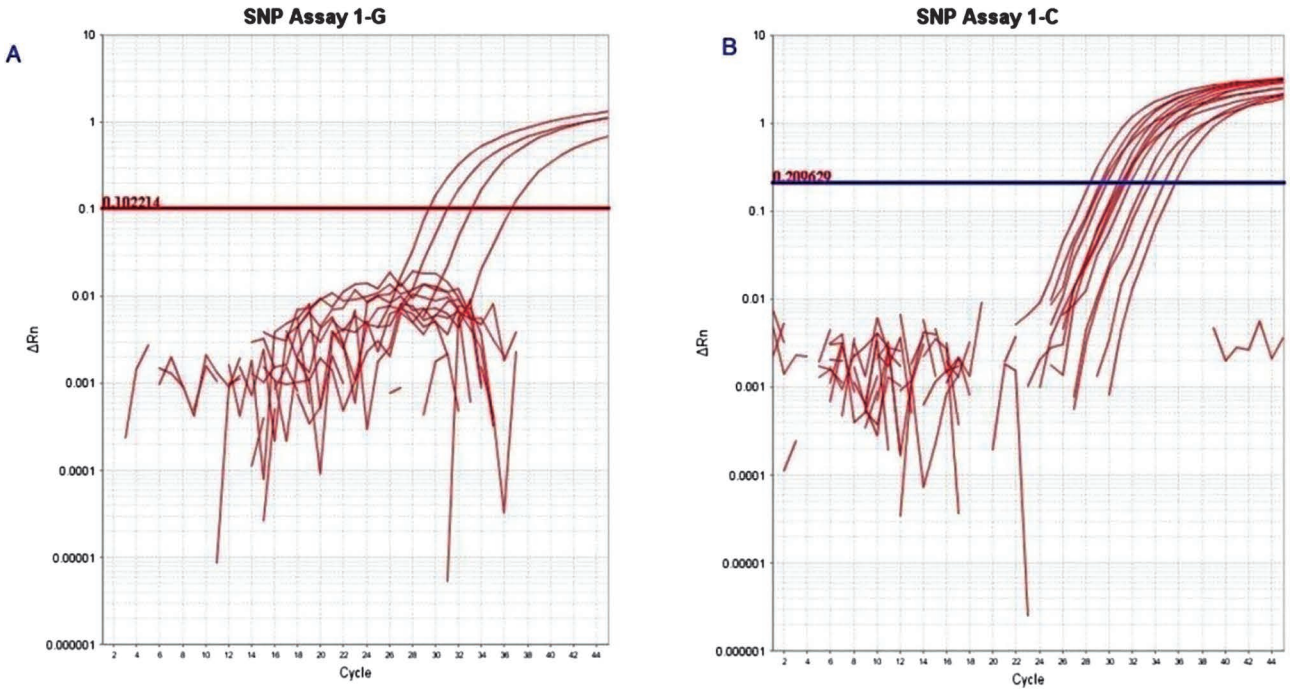

Figure 4. Amplification curves of real-time polymerase chain reaction. The A and B amplification curves (ΔRnvs cycle) show each allele (allele G and allele C) separately in real-time polymerase chain reaction, software version 2.0.1. SNP indicates single nucleotide polymorphism.

According to the kidney biopsy, 66% of the patients had minimal change nephrotic syndrome, 20% had focal segmental glomerulosclerosis, and 14% had membrano-proliferative glomerulosclerosis.

A significant difference between the children with NS and the controls was observed regarding protein-creatinine ratio, albumin, total protein, blood urea nitrogen, creatinine, hemoglobin, and cholesterol (Table 1).

The IL-6-G174C exhibited a significantly different genotype distribution among the children with NS compared with the controls (GG versus CC, $P = .02$; GG versus GC, $P = .003$; OR, 5.83; 95% CI, 1.64 to 20.70; as well as alleles distribution of G versus C, $P < .001$; OR, 7.57; 95% CI, 2.28 to 25.17). With regard to TNF-α-G308A genotype, there was no significant difference in genotype distribution of the children with NS compared with the controls, but a significant difference was observed at the alleles level (G versus A, $P = .04$; OR, 2.66; 95% CI, 1.00 to 7.10; Table 2).

There was a significant difference between the steroid-sensitive and steroid-resistant patients regarding age, hypertension, hematuria, pathology examination findings, protein-creatinine ratio, and serum levels of total protein, urea, and cholesterol (Table 3). Comparing the steroid-resistant group with the steroid-sensitive group, significant association was found at genotypic level in case of IL-6-G174C (GG versus CC, $P = .03$; OR, 5.52; 95% CI, 1.39 to 21.89), but no association was found regarding GG versus GC. At the allelic level of IL-6-G174C, there was no significant association either. The TNF-α-G308A, AA genotype and A allele distribution were higher in the steroid-sensitive than the steroid-resistant group, though not reaching a statistical significance ($P = .72$ and $P = .55$, respectively; Table 4).

Table 1. Demographic and Laboratory Data of Children With Idiopathic Nephrotic Syndrome (INS) and Healthy Controls*

Parameter	INS Patients (n = 100)	Controls (n = 30)	*P*
Age, y	7.51 ± 3.12 (5 to 15)	8.47 ± 3.80 (5 to 15)	.75
Sex			
Male	63 (63.0)	19 (63.3)	
Female	37 (37.0)	11 (36.7)	.97
Urine protein-creatinine ratio, mg/mg	2.57 ± 0.79 (1.4 to 4.7)	0.43 ± 0.19 (0.2 to 0.8)	< .001
Serum albumin, g/dL	2.31 ± 0.44 (1.3 to 3.2)	4.21 ± 0.48 (3.6 to 5.2)	< .001
Total protein, g/dL	4.13 ± 0.99 (1.0 to 6.3)	6.86 ± 0.50 (6.0 to 7.8)	< .001
Blood urea, mg/dL	23.34 ± 8.11 (13 to 45)	16.9 ± 7.2 (11 to 34)	< .001
Serum creatinine, mg/dL	0.66 ± 0.26 (0.2 to 1.3)	0.40 ± 0.12 (0.2 to 0.7)	< .001
Blood hemoglobin, g/dL	11.23 ± 1.22 (7.8 to 14.1)	12.77 ± 0.59 (12 to 14)	< .001
Serum cholesterol, mg/dL	393.29 ± 72.67 (234 to 530)	114.90 ± 16.18 (98 to 160)	< .001

*Values are mean ± standard deviation (range), except for sex distribution, which is frequency (percentage).

Table 2. Frequency of Genotypes and Allele Distribution Among Children With Idiopathic Nephrotic Syndrome (INS) and Healthy Controls

Genotype	INS Patients (n = 100)	Controls (n = 30)	*P*	Odds Ratio (95% Confidence Interval)
IL-6-G174C				
GG	54 (54)	27 (90)	…	Reference
GC	35 (35)	3 (10)	.003	5.83 (1.64 to 20.7)
CC	11 (11)	0 (0.0)	.02	…
IL-6 allele				
G	143 (71.5)	57 (95.0)	…	Reference
C	57 (28.5)	3 (5.0)	< .001	7.57 (2.28 to 25.17)
TNF-α-G308A				
GG	72 (72.0)	25 (83.3)	…	Reference
GA	17 (17.0)	5 (16.7)	.77	1.18 (0.39 to 3.53)
AA	11 (11.0)	0 (0.0)	.06	…
TNF-α allele				
G	161 (80.5)	55 (91.7)	…	Reference
A	39 (19.5)	5 (8.3)	.04	2.66 (1.0 to 7.1)

Table 3. Demographic and Laboratory Data of Steroid-Sensitive and Steroid-Resistant Children

Parameter	Steroid-Sensitive Patients (n = 72)	Steroid-Resistant Patients (n = 28)	*P*
Age, y	5.69 ± 0.91 (5 to 10)	12.18 ± 1.42 (10 to 15)	< .001
Sex			
Male	43 (59.7)	20 (71.4)	
Female	29 (40.3)	8 (28.6)	.28
Generalized edema	72 (100)	28 (100)	> .99
Hypertension	11 (15.3)	21 (75.0)	< .001
Hematuria	3 (4.2)	20 (71.4)	< .001
Kidney biopsy			
Minimal change	59 (81.9)	7 (25.0)	
Focal segmental glomerulosclerosis	8 (11.1)	12 (42.9)	
Membranoproliferative glomerulosclerosis	5 (6.9)	9 (32.1)	< .001
Urine protein-creatinine ratio, mg/mg	2.22 ± 0.48 (1.4 to 3.5)	3.46 ± 0.73 (1.7 to 4.7)	< .001
Serum albumin, g/dL	2.34 ± 0.42 (1.3 to 3.2)	2.24 ± 0.48 (1.3 to 3.1)	.28
Total protein, g/dL	4.45 ± 0.81 (2.7 to 6.3)	3.31 ± 0.99 (1 to 5.1)	< .001
Blood urea, mg/dL	20.0 ± 4.79 (13 to 32)	31.93 ± 8.64 (14 to 45)	< .001
Serum creatinine, mg/dL	0.60 ± 0.23 (0.2 to 1.2)	0.82 ± 0.26 (0.3 to 1.3)	< .001
Blood hemoglobin, g/dL	11.30 ± 1.22 (8.7 to 14.1)	11.07 ± 1.22 (7.8 to 13.2)	.40
Serum cholesterol, mg/dL	369.01 ± 63.28 (234 to 520)	455.71 ± 56.79 (290 to 530)	< .001

The participants with mutant alleles of IL-6 (C) in different combinations were significantly associated with diseased subjects in comparison to the controls. While combined analysis of IL-6 and TNF-α genotypes among the steroid-sensitive and steroid-resistant patients did not show any significant association with disease progression (Table 5).

Table 4. Genotypes and Allele Distribution Among Steroid-Sensitive and Steroid-Resistant Children

Genotype	Steroid-Sensitive Patients (n = 72)	Steroid-Resistant Patients (n = 28)	*P*	Odds Ratio (95% Confidence Interval)
IL-6-G174C				
GG	41 (56.9)	13 (46.4)	…	Reference
GC	27 (37.5)	8 (28.6)	.89	0.93 (0.34 to 2.56)
CC	4 (5.6)	7 (25.0)	.03	5.52 (1.39 to 21.89)
IL-6 allele				
G	109 (75.7)	34 (60.7)	…	Reference
C	35 (24.3)	22 (39.3)	.06	1.91 (0.99 to 3.66)
TNF-α-G308A				
GG	51 (70.8)	21 (75.0)	…	Reference
GA	12 (16.7)	5 (17.9)	> .99	0.99 (0.31 to 3.15)
AA	9 (12.5)	2 (7.1)	.72	1.85 (0.37 to 9.31)
TNF-α allele				
G	114 (79.2)	47 (83.9)	…	Reference
A	30 (20.8)	9 (16.1)	.55	1.37 (0.61 to 3.12)

Table 5. Combined Analysis of Genotypes Among Children With Idiopathic Nephrotic Syndrome (INS) Versus Healthy Controls and Steroid-Sensitive Versus Steroid-Resistant Children

IL-6-G174C and TNF-α-G308A Genotypes*	INS Patients	Controls	*P*	Odds Ratio (95% Confidence Interval)	Steroid-Sensitive Patients	Steroid-Resistant Patients	*P*	Odds Ratio (95% Confidence Interval)
IL-6 (1) and TNF-α (1)	42 (42)	23 (76.7)	…	Reference	32 (44.4)	10 (35.7)	…	Reference
IL-6 (1) and TNF-α (0)	12 (12)	4 (13.3)	.56	1.64 (0.48 to 5.68)	9 (12.5)	3 (10.7)	> .99	0.94 (0.21 to 4.15)
IL-6 (0) and TNF-α (1)	30 (30)	2 (6.7)	.002	8.21 (1.8 to 37.52)	19 (26.4)	11 (39.3)	.30	0.54 (0.19 to 1.51)
IL-6 (0) and TNF-α (0)	16 (16)	1 (3.3)	.02	8.76 (1.09 to 70.37)	12 (16.7)	4 (14.3)	> .99	0.94 (0.25 to 3.57)

*Zero indicates mutant and heterozygous; and 1, wild-type genotype.

DISCUSSION

Response to glucocorticoid therapy is the major determinant of prognosis in INS. Therefore, we studied the association between cytokine genes polymorphisms (IL-6-G174C and TNF-α-G308A) and the response to glucocorticoid therapy in steroid-sensitive and steroid-resistant NS patients compared to healthy individuals. This study revealed that IL-6-G174C CC genotype and the C allele were significantly more frequent among the patients group than the control group. The IL-6-G174C CC genotype was also specifically more frequent among the steroid-resistant group than the steroid-sensitive group; therefore, it may be considered responsible for progression as well as for steroid resistance.

These results are in agreement with the results of 2 studies on the Indian population done by Tripathi and colleagues and Jafar and colleagues, who reported that IL-6-G174C CC genotype and C allele rates were significantly higher among their patient groups than the controls, and that the CC genotype rate was significantly higher among the steroid-resistant group than the steroid-sensitive group.[12,13]

Interleukin-6 is a pleiotropic cytokine with potent biological effects, including stimulation of B and T lymphocytes and induction of the hepatic acute phase response. Since IL-6 is rapidly cleared from the circulation and is not stored in cells, its presence in the blood requires precisely regulated gene transcription and translation. Therefore, genetic variants in regulatory regions of IL-6 might determine individual differences in IL-6 serum concentrations during immune-mediated tissue injury.[14-16] Indeed, clinical studies have demonstrated an association between the IL-6 promoter polymorphism, -174 G>C, with IL-6 serum concentrations and the outcome of various inflammatory diseases.[17-19] Sutherland and coworkers found that an IL-6 haplotype carrying the -174C allele was associated with organ dysfunction in critically ill patients with systemic inflammatory response syndrome.[20,21]

With regards to TNF-α-G308A genotype, there was no significantly different genotype distribution; this is in agreement with Kim and colleagues, who found no significant difference between INS patients and their control group,[22] but contradictory to Jafar and colleagues, who found that AA genotype was significantly more common in the patients compared to the controls.[23] In our study, the A allele exhibited a significant increase in the patients compared to the controls, and this is in agreement with Jafar and colleagues' report in 2011.[23] The TNF-α-G308A AA genotype and A allele distributions were more frequent in our steroid-sensitive than the steroid-resistant group, though not reaching statistical significant. However, contradictory to our results, Jafar and colleagues found that the frequency of A allele was higher in steroid-resistant group than in steroid-sensitive group.[23]

Tumor necrosis factor-α is a pro-inflammatory multifunctional cytokine produced by macrophages.[24] It plays an important role in the regulation of immune response since its increase after traumatic injury generates a cytokine cascade resulting in activation, proliferation, and hypertrophy of mononuclear and phagocytic cells.[25] The biological activities of TNF-α and the fact that its gene is located within the major histocompatibility complex have suggested that polymorphisms in this locus may be associated with autoimmune, infectious, and neoplastic disorders.[26] The G>A transition polymorphism at 308 position of the TNF-α gene is important for its expression, since it is situated within the binding site of the AP-2 repressive transcription factor.[27] The TNF-α A allele is associated with a higher level of TNF-α transcript, justified by the greater potency of the promoter region to activate the transcription.[27,28] The presence of A allele has been found to correlate with enhanced spontaneous or stimulated TNF-α production in vitro and in vivo.[29] The presence of TNF-α A allele among steroid-sensitive patients rather than steroid-resistant patients may play a role in immunomodulation and response to glucocorticoid therapy among the steroid-sensitive group, and it may be a predictor of good response to therapy.

CONCLUSIONS

The IL-6-G174C and TNF-α-G308A polymorphisms may affect susceptibility to INS and might affect steroid response in INS patients. Individuals with mutant alleles of IL-6 (C) in different combinations were significantly associated with INS.

CONFLICT OF INTEREST

None declared.

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