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ORIGINAL CONTRIBUTIONS

DOES CYTOKINE GENE POLYMORPHISM AFFECT STEROID RESPONSES IN IDIOPATHIC NEPHROTIC SYNDROME?

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ABSTRACT

BACKGROUND: *Immunological responses may be possibly involved in the pathogenesis of idiopathic nephrotic syndrome (INS). Cytokines act as a potent immunomodulator. Pathogenesis of INS is associated with Th1 and Th2 cytokines imbalance.* ***AIMS, SETTINGS AND DESIGN:*** *We have investigated the association of IL-4, IL-6, and TNF-α gene polymorphisms and analyzed the data to evaluate the effect of these polymorphisms on the pathogenesis and clinical course of INS.* ***MATERIALS AND METHODS:*** *One hundred fifty children with INS were selected. Children were analyzed for IL-4, IL-6, and TNF-α gene polymorphisms by using polymerase chain reaction and restriction fragment length polymorphism.* ***STATISTICAL ANALYSIS USED:*** *Chi-square test was used for different comparisons. The synergistic effects of IL-4, IL-6, and TNF-α gene polymorphisms were evaluated by using logistic regression analysis.* ***RESULTS AND CONCLUSIONS:*** *We compared the steroid-resistant (SR) and steroid-responsive (SS) groups. Our results showed strong association of IL-6 -G174C, and IL-4 -C590T at genotypic level (P = 0.0121, OR = 14.71, 95% CI = 1.59-136.46; and P = 0.0386, OR = 7.29, 95% CI = 1.26-41.69). TNF-α revealed a strong association at genotypic level (P = 0.0121, OR = 14.71, 95% CI = 1.59-136.46), as well as at allelic level (P = 0.0433, OR = 2.251, 95% CI = 1.09-4.66), demonstrating that it may be considered one of the genetic risk factors affecting the steroid response in INS patients. The GG genotype of IL-6 -G174C, TT genotype of IL-4 -C590T, and AA genotype of TNF-α -G308A cytokine gene polymorphisms may be causative factors for nonresponsiveness towards steroid therapy among INS children.*

Key words: *Idiopathic nephrotic syndromes, IL-4, IL-6, TNF-α, steroid resistant, steroid responsive*

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most common glomerular disorder of childhood, with 1 to 2 new cases per 100,000 children per year. INS is characterized by the presence of proteinuria, which is associated with hypoalbuminemia and hyperlipidemia. Some patients with INS do not respond to glucocorticoids, i.e., they are steroid resistant (SR). Most of the children with INS are steroid sensitive (SS), i.e., they achieve remission following steroid treatment. However, some of the children with steroid sensitive nephrotic syndrome (SSNS) will undergo relapse once or more. About 50% of the relapsers are either frequent relapsers or develop steroid dependence.[1] The mechanisms underlying these different responses to steroids are not very well understood; however, there may be certain genetic factors involved for these differential drug responses.

Histology of childhood INS is most commonly represented by minimal-change nephrotic syndrome (MCNS) followed by focal segmental glomerulosclerosis (FSGS) and rarely by membrano-proliferative glomerulonephritis (MPGN) and membranous glomerulonephritis (MGN). Children with FSGS, MPGN, and MGN usually have a poorer response to glucocorticoids.[1]

Idiopathic nephrotic syndrome was proposed to be a disorder of T-cell dysfunction.[2] The mechanisms by which T-cells affect the course of the disease remain elusive. However, there may be circulating factors released from activated T-cells which may affect the pathogenesis of the disease. Th1 cells produce IL-2, IFN-γ, and tumor necrosis factor-beta (TNF-β); and promote both macrophage activation resulting into DTH, and production of complement-fixing and opsonizing antibodies. Th2 cells, which synthesize IL-4, IL-5, IL-6, IL-10, and IL-13, provide optimal help for antibody production; and promote both mast cell growth and eosinophil differentiation and activation, resulting in humoral responses.[2,3] INS is considered to be an immune-mediated disease;[4] however, the contribution of Th1 and Th2 cytokines is a matter of debate. Several cytokines are considered prime candidates for the role of mediators of INS.[5] However, such data is missing in the literature from the Indian subcontinent.

The present study was conducted to investigate the possible association between IL-4, IL-6, and TNF-α gene polymorphisms and their effect on steroid responses in 150 children with idiopathic nephrotic syndrome. We have also tried to see the synergistic effects of various cytokine SNP polymorphisms; especially the constellation of TNF-α and IL-6 genetic variants might predispose some INS patients to differential response to inflammation, and also to the steroids.

MATERIALS AND METHODS

Patients

A prospective analysis was done in 150 children (male=106, female=44) with idiopathic nephrotic syndrome. Their mean age at diagnosis was 4.8 ± 3.4 years (range, 1-18 years), They presented to the OPD at our hospital from January 2005 to January 2008. All patients were diagnosed according to the International Study of Kidney Disease in Children (ISKDC) criteria as defined

in our earlier study,[6] and all of them had developed nephrotic syndrome before the age of 18 years. Patients with poor compliance and not on regular follow-up were excluded from the study. Patients were categorized according to their clinical response to glucocorticoids into (a) steroid-resistant (SR, no achievement of remission despite treatment with prednisolone at 60 mg/m^2/d for 6-8 weeks) patients; (b) steroid-dependent (SD, 2 consecutive relapses occurring during steroid treatment or within 2 weeks of steroid withdrawal) patients; (c) frequent relapsers (FR, 4 or more relapses within a 12-month period in a steroid responder); and (d) infrequent relapsers (IFR, all others). The steroid-responsive group was compared with the steroid-resistant group in terms of genotypes and allele frequencies of IL-4, IL-6, and TNF-α.

All children were subjected to detailed history-taking and physical examination. In addition, following biochemical tests were done to confirm the diagnosis of nephrotic syndrome: serum creatinine, total protein, albumin, cholesterol, triglycerides, urinary routine microscopy examination, urine protein and creatinine ratio in a spot sample Based upon the difference in steroid responsiveness, renal biopsies were carried out in most of the steroid-resistant cases and some of the cases of SD and FR as per availability of consent. The characteristics of INS patients included in the present study are shown in Table 1. An informed written consent was obtained from all participants. The study was approved by the ethical committee of SGPGIMS and Department of Biotechnology, Government of India.

Blood collection and DNA extraction

Blood samples were obtained to measure serum biochemical parameters and lipid profiles in the morning after fasting of 8 hours. Three milliliters of venous blood sample was collected in EDTA vials. Genomic DNA was extracted from the whole blood using a commercially available genomic DNA purification kit (Qiagen kit).

Table 1: Characteristics of patients with idiopathic nephrotic syndrome

	No biopsy (n=59)	*MCNS (n=40)*	*FSGS (n=35)*	*MPGN (n=14)*	*MGN (n=2)*	*All INS (n=150)*
Infrequent relapsers (IFR)	28(100)	-	-	-	-	28(18.7)
Frequent relapsers (FR)	13(30.9)	17(40.5)	09(21.4)	03(7.1)	0	42(28.0)
Steroid dependent (SD)	11(24.4)	16(35.6)	12(26.7)	05(11.1)	01(2.2)	45(30.0)
Steroid dependent (SR)	07(20.0)	07(20.0)	14(40.0)	06(17.1)	01(2.9)	35(23.3)

MCNS= Minimal-change nephrotic syndrome, FSGS= Focal segmental glomerulosclerosis, MPGN= Membrano-proliferative glomerulonephritis, MGN= Membranous glomerulonephritis, Figures in parentheses are in percentage

Table 2: Genotyping conditions for IL-6G174C, IL-4C590T, and TNF-αG308A polymorphisms studied in children with INS (n = 150) and control (n = 569) samples

Marker	*Primer sequence*	*Primer annealing conditions*	*RE's*	*Alleles*	*Product size (bp)*	*Reference*
IL-4 -C590T	*F- 5'ACTAGGCCTCACCTGATACG3'* *R- 5'GTTGTAATGCAGTCCTCCTG3'*	*58°C for 45 s*	*BsmF1*	*C* *T*	*192 and 60* *252*	*10*
TNF-α -G308A	*F- 5'AGGCAATAGGTTTTGAGGGCCAT3'* *R- 5'TCCTCCCTGCTCCGATTCCG3'*	*59.3°C for 60 s*	*Nco1*	*A* *G*	*107* *87 and 20*	*25*
IL-6 -G174C	*F- 5'GGAGTCACACACTCCACCT3'* *R- 5'GTGGGGCTGATTGGAAACC3'*	*64.2°C for 60 s*	*SfaN1*	*G* *C*	*532* *474 and 58*	*17*

RE's =Restriction enzymes, bp =base pair

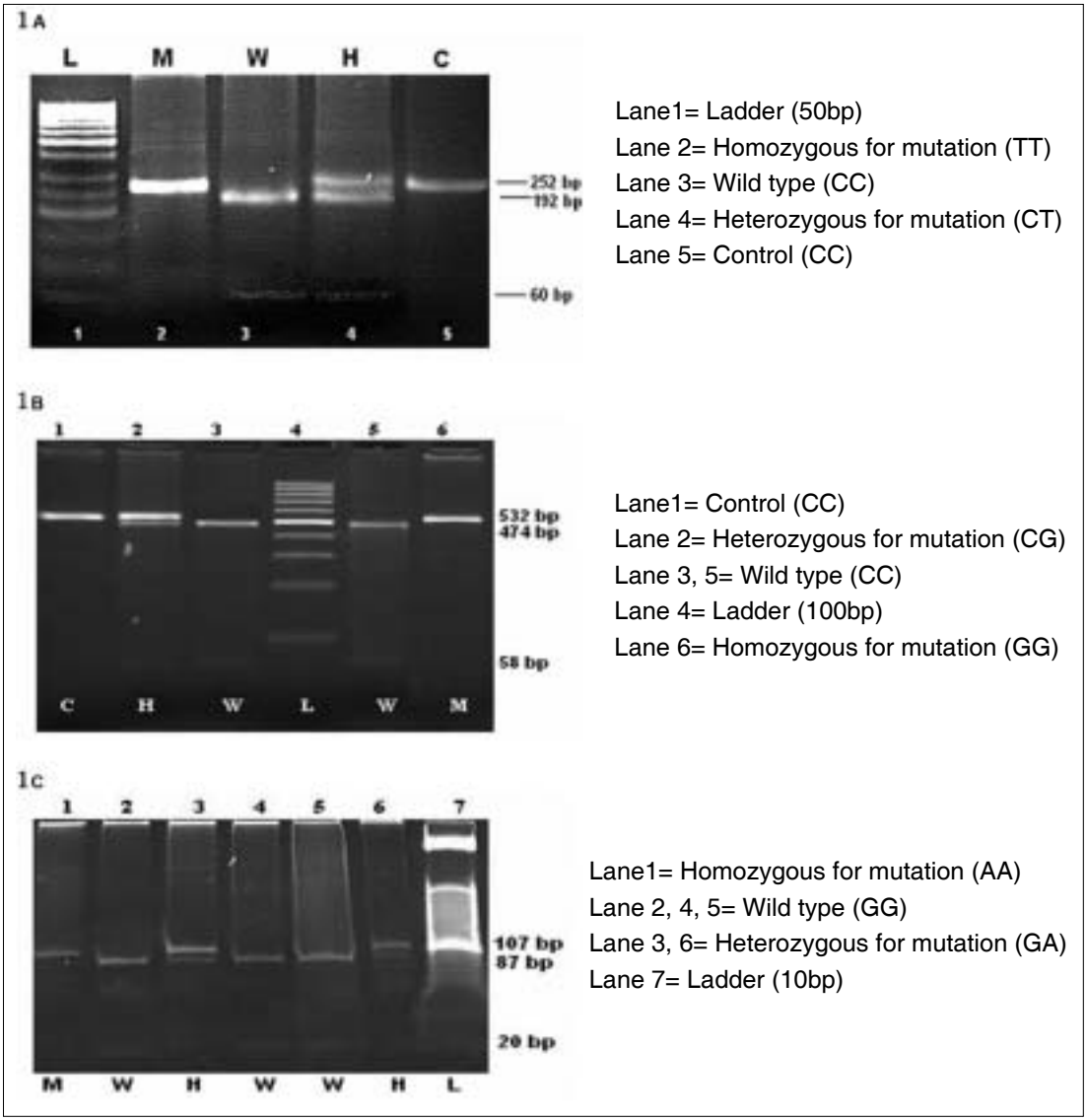

Figure 1: (A) IL-4 -C590T, PCR amplicons electrophoresis after restriction digestion with enzyme BsmF1, (B) IL-6 -G174C, PCR amplicons electrophoresis after restriction digestion with enzyme SfaN1, (C) TNF-α -G308A, PCR amplicons electrophoresis after restriction digestion with enzyme Nco1

Analysis of the IL-6, IL-4, and TNF-α genotype

The genotyping was done by PCR-restriction fragment length polymorphism [Figures 1A-1C]. The genotyping protocol for the detection of IL-4, IL-6, and TNF-α gene polymorphisms is shown in Table 2.

To improve the genotyping quality and validation, all mutant and heterozygous

Table 3: Distribution of IL-6 -G174C, IL-4 -C590T, and TNF-α -G308A genotypes/alleles among steroid-responsive and steroid-resistant patients

Variants	*SS=115 (76.7%)*	*SR=35 (23.3%)*	*OR (95% CI)*	*P-value (< 0.05)*
IL-6 G174C				
CC	65 (56.5)	17 (48.6)	1.38 (0.64-2.94)	0.5265
GC	49 (42.6)	14 (40.0)	1.11 (0.52-2.41)	0.9376
GG	01 (0.9)	04 (11.4)	14.71 (1.59-136.46)	0.0121*
Allele C	179 (77.8)	48 (68.6)	1.61 (0.89-2.91)	0.1553
Allele G	51 (22.2.)	22 (31.4)		
IL-4 C590T				
CC	71 (61.7)	22 (62.8)	0.95 (0.44-2.09)	0.9050
TC	42 (36.6)	09 (25.8)	1.66 (0.71-3.88)	0.3280
TT	02 (1.7)	04 (11.4)	7.29 (1.26-41.69)	0.0386*
Allele C	184 (80.0)	53 (75.7)	1.28 (0.68-2.42)	0.5463
Allele T	46 (20.0)	17 (24.3)		
TNF-α G308A				
GG	93 (80.9)	25 (71.5)	1.69 (0.71-4.03)	0.3380
AG	21 (18.2)	06 (17.1)	1.08 (0.39-2.93)	0.8802
AA	01 (0.9)	04 (11.4)	14.71 (1.59-136.46)	0.0121*
Allele G	207 (90.0)	56 (80.0)	2.25 (1.09-4.66)	0.0433*
Allele A	23 (10.0)	14 (20.0)		

*Significant value ($P < 0.05$), SS: Steroid-responsive, SR: Steroid-resistant

samples were re-genotyped blindly and results were noted only for those samples which were reproducible with no discrepancy. Genotyping of 20% of the samples was confirmed by DNA sequencing.

Statistical analysis

Sample size for INS children was calculated by a statistician of our institute. Statistical analyses for the genotypic and allelic frequencies were performed using chi-square test (Graphpad Software version 2.0). To evaluate the synergistic effects of IL-4, IL-6, and TNF-α gene polymorphisms, we did the logistic regression analysis by using SPSS software (version 15). Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least one copy of the test allele divided by the total number of individuals. *P* values less than 0.05 were considered statistically significant. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Yates correction was applied wherever required.

RESULTS

Allele and genotypes frequencies of IL-6, IL-4 and TNF-α were compared between the SS and SR groups.

Among 150 INS patients, 115 (76.7%) were steroid responsive (SS) and 35 (23.3%) were steroid resistant (SR). The allele and genotype distribution of IL-6, IL-4, and TNF-α gene polymorphisms in SS and SR groups are shown in table 3. There was a strong association between IL-6 and IL-4 at the genotypic level in SS and SR groups (P = 0.0121, OR = 14.71, 95% CI = 1.59-136.46; and P = 0.0386, OR = 7.29, 95% CI = 1.26-41.69) respectively. Whereas TNF-α showed a strong association at genotypic level (P = 0.0121, OR = 14.71, 95% CI = 1.59-136.46) as well as at allelic level (P = 0.0433, OR = 2.25, 95% CI = 1.09-4.66) in SS and

SR groups. Further synergistic effects of IL-4, IL-6, and TNF-α gene polymorphisms were evaluated, and no association of combined genotypes with SS and SR groups were observed [Table 4].

An attempt was made to correlate the allele frequency of INS with onset of the disease. We divided the patient population into 2 age groups: ≤5 years and >5 years. No significant difference was found between the 2 groups when age of onset and co-occurrence of the various genes and their genotypes were studied [Table 5].

DISCUSSION

In the present study we have investigated an association between cytokines gene polymorphisms in 150 children with INS and their response to steroids. Children with INS were genotyped for the IL-4, IL-6, and TNF-α gene polymorphisms. Our results demonstrated that IL-6 -G174C and TNF-α -A308G polymorphism may be one of the genetic risk factors for INS and may affect steroid response among the north Indian population. Our results revealed that there was a strong association between IL-4 and IL-6 at genotypic level in SS and SR groups (P = 0.0386 and P = 0.0121). Interestingly TNF-α showed a strong association at genotypic level (OR = 14.71), as well as at allelic level (OR = 2.25) in both the SS and SR groups, which demonstrates that this may be one of the risk factors for INS and may affect steroid response.

There are reports showing cytokine genes polymorphism to be associated with the development and severity of inflammatory diseases.[7-9]

Minimal-change nephrotic syndrome (MCNS)

Table 4: Combined analysis of IL-6G174C, IL-4C590T, and TNF-α G308A genotypes among INS patients

Genotype	*SS=115 (76.7%)*	*SR=35 (23.3%)*	*P-value*	*OR (95 % CI)*
Double: IL-4 +IL-6				
IL-4 (1) and IL-6 (1)	37 (32.2)	11 (31.4)	0.9340	1.04 (0.46-2.34)
IL-4 (1) and IL-6 (0)	34 (29.6)	11 (31.4)	0.5935	0.73 (0.33-1.66)
IL-4 (0) and IL-6 (1)	28 (24.3)	06 (17.2)	0.5087	1.56 (0.58-4.13)
IL-4 (0) and IL-6 (0)	16 (13.9)	07 (20.0)	0.5437	1.55 (0.58-4.13)
Double: IL-4 +TNFα				
IL-4 (1) and TNFα (1)	56 (48.7)	14 (40.0)	0.4781	1.42 (0.66-3.07)
IL-4 (1) and TNFα (0)	15 (13.0)	08 (22.9)	0.2530	1.98 (0.76-5.15)
IL-4 (0) and TNFα (1)	37 (32.2)	11 (31.4)	0.9340	1.04 (0.46-2.34)
IL-4 (0) and TNFα (0)	07 (6.1)	02 (5.7)	0.9352	1.07 (0.21-5.40)
Double: IL-6 +TNFα				
IL-6 (1) and TNFα (1)	53 (46.1)	11 (31.4)	0.1802	1.87 (0.84-4.16)
IL-6 (1) and TNFα (0)	12 (10.4)	06 (17.1)	0.4399	1.78 (0.61-5.14)
IL-6 (0) and TNFα (1)	40 (34.8)	14 (40.0)	0.7174	1.25 (0.57-2.72)
IL-6 (0) and TNFα (0)	10 (8.7)	04 (11.4)	0.8769	1.36 (0.39-4.62)
Triple: IL-4 + IL-6 + TNFα				
IL-4 (1) + IL-6 (1) + TNFα (1)	29 (25.2)	06 (17.1)	0.4468	1.63 (0.61-4.32)
IL-4 (0) + IL-6 (0) + TNFα (0)	03 (2.6)	01 (2.9)	0.9363	1.09 (0.11-10.90)
IL-4 (0) + IL-6 (1) + TNFα (0)	04 (3.5)	01 (2.9)	0.8577	0.82 (0.09-7.56)
IL-4 (0) + IL-6 (1) + TNFα (1)	24 (20.9)	05 (14.3)	0.5358	1.58 (0.55-4.52)
IL-4 (1) + IL-6 (0) + TNFα (0)	07 (6.1)	03 (8.5)	0.8974	1.15 (0.35-5.92)
IL-4 (1) + IL-6 (0) + TNFα (1)	27 (23.5)	08 (22.9)	0.8903	1.19 (0.48-2.90)
IL-4 (1) + IL-6 (1) + TNFα (0)	08 (6.9)	05 (14.3)	0.3142	2.23 (0.68-7.32)
IL-4 (0) + IL-6 (0) + TNFα (1)	13 (11.3)	06 (17.1)	0.5358	1.62 (0.57-4.65)

0 =mutant + heterozygous, 1 =wild type genotype

Table 5: Distribution of IL-6G174C, IL-4C590T, and TNF-αG308A genotypes/alleles among patients according to age

Variants	*Age ≤ 5 96 (64.0%)*	*Age >5 54 (36.0%)*	*OR (95% CI)*	*P- Value (< 0.05)*
IL-6 G174C				
CC	47 (48.9)	35 (64.8)	1.92 (0.97-3.82)	0.0888
CG	46 (48.0)	18 (33.3)	1.84 (0.92-3.68)	0.1184
GG	03 (3.1)	01 (1.9)	1.71 (0.17-16.86)	0.6422
Allele C	140 (72.9)	88 (81.5)	1.63 (0.91-2.92)	0.1269
Allele G	52 (27.1)	20 (18.5)		
IL-4 C590T				
CC	61 (63.5)	32 (59.3)	1.27 (0.63-2.53)	0.6127
TC	32 (33.3)	19 (35.2)	0.92 (0.46-1.86)	0.9599
TT	03 (3.1)	03 (5.6)	1.82 (0.36-9.37)	0.7679
Allele C	154 (80.2)	83 (76.9)	1.46 (0.83-2.57)	0.2466
Allele T	38 (19.8)	25 (23.1)		
TNF-α G308A				
GG	77 (80.2)	41 (75.9)	1.29 (0.58-2.86)	0.6841
AG	17 (17.7)	11 (20.4)	0.84 (0.36-1.96)	0.8545
AA	02 (2.1)	02 (3.7)	1.80 (0.25-13.22)	0.9495
Allele G	171 (89.1)	93 (86.1)	1.31 (0.65-2.67)	0.5687
Allele A	21 (10.9)	15 (13.9)		

in children is frequently associated with allergy and immunoglobulin E production. T helper subtype 2 cytokines, such as interleukin-4 (IL-4), may have an important role in the development of atopy. Some of the studies suggest that genetic variations in IL-4 may be associated with predisposition to MCNS, and partially to the clinical course of MCNS.[10-12] IL-4 production by peripheral Th cells is up-regulated in patients with MCNS and correlated with the severity of proteinuria.[13] Our results demonstrated that IL-4 -C590T SNP influences the prognosis of the disease. We have found that TT genotype was higher in the steroid-resistant group ($P = 0.0386$) as compared to SR.

IL-6 and its genetic variants have been studied in various disorders which involve susceptibility to recurrent infections; thrombophilia; and immunological alterations, such as celiac disease, breast cancer, systemic lupus erythromatosus, and leishmaniasis.[14-17] Though the roles of IL-6 and IL-6 -G174C are not well studied in nephrotic syndrome, their role have been found in mesangial proliferative glomerulonephritis and end-stage renal disease.[18] Our study revealed that GG genotype of IL-6 -G174C is more prevalent in the steroid-resistant group, thus pointing towards a poorer prognosis in children with nephrotic syndrome.

Tumor necrosis factor alpha (TNF-α) is a potent immunomodulator and pro-inflammatory cytokine and has been implicated in many pathological processes. Polymorphism at position -308 of the TNF-α promoter, representing G to A base transitions, has been linked to increased TNF transcription.[19,20] Earlier studies have shown increase of TNF-α synthesis and gene expression in patients with idiopathic nephrotic syndrome and focal glomerular sclerosis.[21,22] Since TNF-α activates NF-κB and angiotensinogen[23,24] hence steroid induced immuno- suppression is critically dependent upon inhibition of NF-κB Elevation of TNF-α has been found in the plasma and urine of patients with INS.[25] We studied the

genotype of TNF-α G308A in patients with nephrotic syndrome and its relation with steroid responsiveness. AA genotype of TNF-α genotype was found to be significantly higher in the steroid-resistant group, along with a higher 'A' allele frequency (20.0%). The transcriptional and post-transcriptional alterations of NF-kappaB/I-kappaBalpha in nephrotic syndrome[26] can be a potential pathway for TNF-α action in determining steroid responsiveness; however, this needs further evaluation for determining the proper therapeutic approach in children with nephrotic syndrome.

CONCLUSION

We may state that the studied polymorphism might affect steroid response in INS patients. Further, atopy-related pathway (involving IL-4) may be a good modulator for the prognosis and steroid resistance in nephrotic syndrome; whereas susceptibility to infection (involving IL-6) and markers of inflammation like TNF-α seems to determine, at least partly, the prognosis of nephrotic children, especially their response to steroid treatment. However, the role of other genetic and environmental factors cannot be ruled out. Hence further studies are required to precisely define the biochemical mechanism of action of these genes, which can help in the development of methods for the prediction, prevention, and treatment of idiopathic nephrotic syndrome.

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