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Association of Cytokine Genes Polymorphisms and the Response to Corticosteroid Therapy in Children with Idiopathic Nephrotic Syndrome: A pilot study in Egypt

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ABSTRACT

The pathogenesis of idiopathic nephrotic syndrome (INS) is associated with Th1 and Th2 cytokines imbalance. Cytokines act as mediators of inflammation in childhood NS. The objectives are to investigate the role of cytokine genes polymorphisms (IL6-G174C, IL4-C590T, and TNFα-G308A and the response to corticosteroid therapy in children with INS in Egypt. 90 children were included in this study, they divided into 3 groups: 30 children with steroid sensitive nephrotic syndrome (SSNS), 30 children with steroid resistant nephrotic syndrome (SRNS) and 30 age and sex matched healthy control children. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) were used to assess IL6-G174C, IL4-C590T, and TNFα-G308A polymorphisms. A significant association was found between the CC genotype and the C allele in IL6-G174C and INS group when compared to the control group (P = 0.003 and 0.009, OR = 4.3 and 2.67 respectively), also, they were significantly associated with SRNS compared to SSNS groups (P = 0.008 and 0.000, OR = 4.93 and 5.82 respectively). Also, TNF-α G308A AA genotype A allele were significantly associated with INS group compared to the control group (P = 0.045 and 0.014, OR = 3.5 and 3.51 respectively) and on comparing SRNS to SSNS groups TNF-α G308A A allele was only significantly associated with SSNS group (p = 0.032, OR = 2.83). IL4-C590T showed no significant difference in the genotypes or the allele distribution between the studied groups. In conclusion, IL6-G174C and TNFα-G308A polymorphisms may affect response to steroid therapy in childhood INS.

Keywords: Idiopathic nephrotic syndromes, IL-4, IL-6, TNF-α gene polymorphism, steroid response.

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INTRODUCTION

Nephrotic syndrome (NS) is characterized by heavy proteinuria, hypoalbuminemia, hypercholesterolemia and edema (Safaei and Maleknejad, 2010). About 90% of cases of NS in children are idiopathic (INS), and the remaining 10% is secondary to systemic diseases including collagen vascular diseases, and infections. About 85% of childhood INS have minimal change nephrotic syndrome (MCNS) and the remaining 15% have other histopathological types (Gbadegesin and Smoyer, 2008).

Steroid responsiveness is the major determinant of prognosis in INS; approximately 85 to 90% of patients with INS respond to steroid treatment with complete remission of proteinuria, while 10 to 15% have partial or even no response to steroid therapy (Kelsch and Sedman, 1993). The mechanisms underlying the difference in response to steroid therapy in INS are not well understood, genetic factors may be involved in those mechanisms (Tripathi et al., 2008).

Idiopathic nephrotic syndrome is considered a primary immune disease associated with immune-regulatory imbalance between T helper 1 cell (Th1) and T helper 2

cell (Th2) cytokines (Jafar et al., 2011). Patients with MCNS display a defect in delayed type hypersensitivity response, which suggest an abnormal Th1 dependent cellular immunity. Allergic manifestations such as contact dermatitis, rhinitis, and asthma might be observed. Th1 cells produce IL2, IFN-γ and TNF-β, and promote both macrophage activation resulting in delayed type hypersensitivity and production of complement fixing and opsonizing antibodies. While, Th2 cells synthesize IL-4, IL-5, IL-6, IL-10 and IL13 providing optimal help for antibody production, and promote both mast cell growth and eosinophil differentiation and activation causing humoral responses (Fodor et al.,1982).

But Th1/Th2 imbalance alone cannot explain the pathogenesis of INS. Th17 cell, which secretes factors such as IL-17, IL-22 and IL-23, was discovered and implicated in the pathogenesis of inflammatory and autoimmune diseases (Shao et al, 2009; Wang et al, 2013). Th17/IL17 has a key function in the pathogenesis of INS by decreasing the expression of podocalyxin (which is the major constituent of the podocyte protein) inducing podocyte apoptosis, which will further decrease the number of podocytes damage the filtration barrier of the glomerulus causing massive proteinuria (Wang et al., 2013).

There is evidence for enhanced activity of IL-4 in MCNS with increased serum levels, increased production by peripheral blood mononuclear cells in vitro and enhanced expression of IgE receptors (Davey et al., 1998). IL4 is an anti-inflammatory cytokine that exerts immunosuppressive effects on macrophage and suppresses pro-inflammatory cytokine production (Von der Thusen et al., 2003).

Interleukin-6 (IL-6) is a pleiotropic cytokine with a central role in host defense. It has diverse functions including stimulation of the hepatic acute phase response and differentiation or activation of macrophages, B- and T-cells. IL-6 is produced by many different cell types and, although initially thought to be a pro-inflammatory cytokine, it has additional anti-inflammatory and immune-suppressive properties (Müller-Steinhardt et al., 2002).

TNF-α is a potent immunomediator and pro-inflammatory cytokine that has been implicated in the pathogenesis of a large number of diseases (Elahi et al., 2009). It has shown an association with various inflammatory diseases; including glomerulonephritis, ankylosing spondylitis, and multiple sclerosis (Shu et al., 2000; Van der Paardt et al., 2002; Mann et al., 2002).

Functional SNPs within the promoter area of these cytokine genes have been identified in that they influence the gene promoter activities and gene product levels (Komatsu et al., 2005, Tindall et al., 2010).

In this study, we investigated the role of cytokine genes promoter polymorphisms (IL6-G174C, IL4-C590T, and TNFα-G308A and response to steroid therapy in children with Idiopathic Nephrotic syndrome in Egypt.

SUBJECTS AND METHODS

This is a case-control study that was conducted in the period from September 2012 to September 2013. Three groups were included in this study. Group I: 30 patients with steroid sensitive nephrotic syndrome (SSNS) (defined as complete remission of proteinuria with urinary protein / creatinine ratio < 200 mg/g or <1+ of protein on urine dipstick for 3 consecutive days within 4 weeks of daily steroid therapy) (KDIGO, 2012), they were 19 male and 11 female, with mean age ± SD of 8.17 ± 3.33 years. Group II: 30 patients with steroid resistant nephrotic syndrome (SRNS) (defined as absence of remission despite therapy with 4 weeks of daily steroids at a dose of 60 mg/m^2/day) (KDIGO, 2012), they were 12 male and 18 female, with a mean age ± SD of 7.8 ± 4.17 years. Patients were recruited from nephrology department and clinic in Cairo University, Mounira Children's Hospital (Abu El Reesh). Patients with familial, infantile, congenital and secondary NS, or with other autoimmune diseases were excluded from the study. Group III: 30 healthy control subjects; 20 males and 10 females, with mean age ± SD of 5.49 ± 3.34 years. The regimens of treatment of the patients were as follows: patients received prednisone 2 mg/kg/day up to 60 mg/day for 4 weeks during initial attacks. Responsive cases were converted to every other day steroid (40 mg/m^2) and were gradually tapered. Non responders receive three methyl prednisolone pulses (30 mg/kg each) to establish steroid resistance. Steroid resistant cases with focal segmental glomerulosclerosis were started on cyclosporine while other resistant cases received cyclophosphamide first. The study was approved by the Local Ethical Committee and informed verbal consents were obtained from the patient's parents.

Patients were subjected to: full clinical evaluation, including history taking and examination. Laboratory investigations: 5 ml venous blood were collected and divided into 2 tubes; 3 ml on plain vacutainers where sera were separated for routine laboratory investigations and 2 ml on EDTA vacutainers for genomic DNA analysis.

Routine laboratory investigations included BUN, Creatinine, total protein, albumin, cholesterol, ALP, total calcium, phosphorus. Random urine specimens were used for urinary protein/creatinine ratio. Analysis was done on Beckman Coulter CX9PRO auto-analyzer.

Genomic DNA analysis: DNA was extracted from peripheral blood leukocytes using Genomic DNA Qiagen Purification Kit (Qiagen GmbH, Qiagen Strasse1, 40724 Hilden Germany). Determination of IL6-G174C, IL4-C590T, and TNFα-G308A polymorphisms was done using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, using Hybaid thermal cycler Promega Corporation, USA, for PCR. The PCR and RFLP protocols for the three genes are mentioned in Table 1. PCR conditions were initial denaturation at 95°C for 5 min, then 35 cycles of 1 min denaturation at 95°C, 1 min annealing (annealing temperatures are shown on Table 1), then 1 minutes extension at 72°C, followed by final extension at 72°C for 10 min after completing the cycles. The amplified products then digested with 5 units of fast digest restriction enzyme (Table 1) at 37°C for 10 min (supplied by Fermentas, LT- 02241 Vilnius, Lithuania). The digested products then detected in 3.5% agarose gel containing Ethidium Bromide by performing electrophoresis on gel electrophoresis apparatus and visualized by UV Trans-illumination (Promega, USA), (Figures 1, 2 and 3).

Statistical analysis

Chi-square test was used to compare allele and genotype frequency among different studied groups. Quantitative data were

Table 1. PCR-RFLP protocols of IL6-G/C rs1800795, IL4-C/T rs2243250, and TNFα-G/A rs1800629 genes (Tripathi et al, 2008).

Gene polymorphism	Primer sequence	Primer annealing condition	*RE	Allele	Product Size (bp)
IL6–G174C	F-5'GGAGTCACACACTCCACCT3' R-5'GTGGGGCTGATTGGAAACC3'	64°C for 60 S	SfaN1	G C	474 & 58 532
IL4-C590T	F-5'ACTAGGCCTCACCTGATACG3' R-5'GTTGTAATGCAGTCCTCCTG3'	58°C for 60 S	BsmF1	C T	192 & 60 252
TNFα-G308A	F-5'AGGCAATAGGTTTTGAGGGCCAT3' R-5'TCCTCCCTGCTCCGATTCCG3'	59°C for 60 S	Nco1	G A	87 & 20 107

*RE: restriction enzyme, bp: base pair.

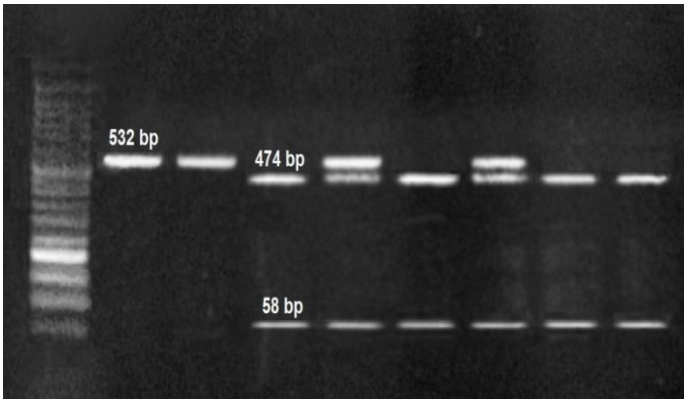

Figure 1. Detection of IL6-G174C by PCR-RFLP, digested with SfaN1 shown on agarose gel electrophoresis. Lane 1 = 50 bp ladder, lanes 2 and 3 = homozygous for the polymorphic allele CC (532 bp), lanes 4, 6, 8 and 9 = homozygous for the wild type (GG) (474, 58 bp), lanes 5 and 7 = heterozygous GC (532, 474 and 58 bp).

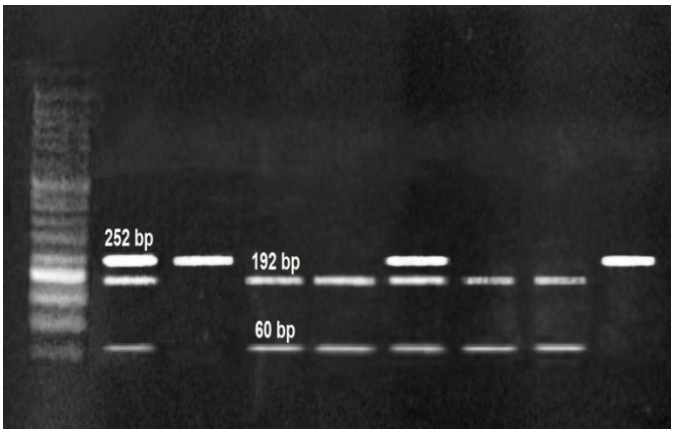

Figure 2. Detection of IL 4-C590T by PCR-RFLP, digested with BsmF1 shown on agarose gel electrophoresis. Lane 1 = 50 bp ladder, lanes 2 and 6 = heterozygous for polymorphism (CT) (252, 192 and 60 bp), lanes 4, 5, 7 and 8 = Wild type (CC) (192 and 60 bp), lanes 3 and 9 = homozygous for polymorphic allele (TT) (252 bp).

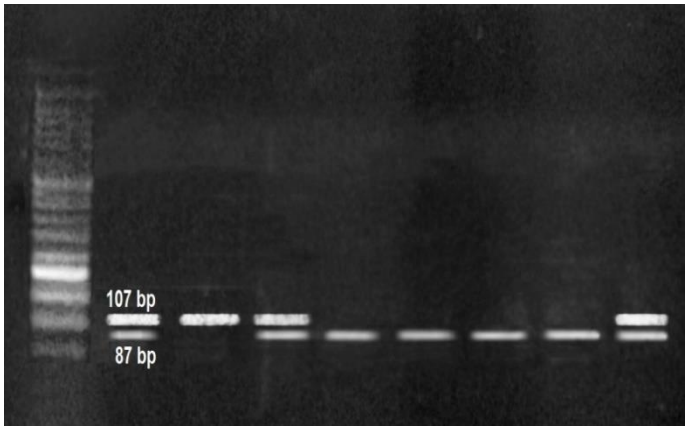

Figure 3. Detection of TNFα –G308A by PCR-RFLP, digested with Nco1 shown on agarose gel electrophoresis. Lane 1 = 50 bp ladder, lanes 2, 4 and 9 = heterozygous for polymorphism (GA) (107, 87 and 20 bp), lane 5, 6, 7 and 8 = Wild type (GG) (87 and 20 bp), lane 3 = homozygous for polymorphic allele (AA) (107 bp).

expressed as mean ± SD and compared using t-test when normally distributed, and as median and range using Mann Whitney U test when not normally distributed. Allele frequency was calculated using the gene counting method (each individual is represented by 2 alleles and allele frequency = number of mutated alleles/ total number of alleles). *P* value <0.05 is considered significant. Odds ratios (OR) and 95% confidence interval (CI) were calculated. Statistics were done using SPSS V.15 software.

RESULTS

The demographic and laboratory data of the studied groups are shown in Table 2. The BUN is significantly higher among the SRNS group compared to both SSNS and control groups, also cholesterol is higher in the 2 patient groups compared to control groups. Total proteins, albumin, and Calcium are higher in the control group compared to the two patient groups.

Comparison of genotypes and allele distribution of IL6-

Table 2. Demographic and laboratory data of the studied groups.

Parameter	SSNS (n = 30)	SRNS (n = 30)	Controls (n = 30)	P-value
*Sex (M/F)%	19/11(63.3/36.7%)	12/18(40/60%)	20/10(66.7/33.3%)	0.076
Age of onset (years)	6.19 (3.042%)	6.44 (2.577%)	-	0.153
*Protein/creatinine ratio (mg/mg creatinine)	2.17(0.1-122.6)	4.65(0.3-162.2)	0.08(0.0-0.2)	0.319
*BUN (mg/dl)	14 (5-48) [a]	20.5 (6-200) [b]	10 (5-19) [a]	0.001
Creatinine (mg/dl)	0.42 ± 0.14	0.97 ± 1.96	0.47 ± 0.138	0.124
Total proteins (g/dl)	5.21 ± 1.59 [a]	5.02 ± 1.24 [a]	7.1 ± 0.51 [b]	0.000
Serum Albumin (g/dl)	2.26 ± 1.16 [a]	2.12 ± 1.01 [a]	3.91 ± 0.47 [b]	0.000
Cholesterol (mg/dl)	391.33±200.91 [a]	366.4±142.24 [a]	95.6 ± 31.27 [b]	0.000
ALP(U/L)	249± 151.07	189.77±97.65	219.8±96.077	0.156
Calcium (mg/dl)	8.3 ± 1.06 [a]	8.38 ± 1.01 [a]	9.58 ± 0.71 [b]	0.000
Hemoglobin (g/dl)	12.43 ± 1.33	11.66 ± 2.03	12.44 ± 1.37	0.109

All data are represented as mean ± standard deviation except (*) albumin/creatinine ratio and BUN are represented as median and average & sex is represented as a %. P-value < 0.05 is significant. SSNS: steroid sensitive nephrotic syndrome, SRNS: steroid resistant nephrotic syndrome. [a] shows significant difference from [b].

Table 3. Genotype and allele frequencies of IL6-G174C, IL4-C590T and TNFα-G308A genes polymorphisms among INS and Control Groups.

SNPs		INS (n = 60)	Control (n = 30)	*P-value	OR (95% CI)
IL6-G174C	GG	26 (43.3%)	23 (76.7%)	Reference	
Genotypes	GC	23 (38.3%)	5 (16.6%)	0.003	4.3
	CC	11 (18.4%)	2 (6.7%)		1.62-11.38
	G	75 (62.5%)	49 (81.6%)	0.009	2.67
Alleles	C	45 (37.5%)	11 (18.3%)		1.21-6.27
IL4-C590T	CC	33 (55%)	13 (43.3%)	Reference	
Genotypes	CT	22 (36.7%)	12 (40%)	0.398	0.63
	TT	5 (8.3%)	5 (16.7%)		0.23-1.65
	C	88 (73.3%)	38 (63.3%)	0.168	0.63
Alleles	T	32 (26.7%)	22 (36.7%)		0.31-1.29
TNFα-G308A	GG	39 (65%)	26 (86.7%)	Reference	
Genotypes	GA	13 (21.7%)	3 (10%)	0.045	3.5
	AA	8 (13.3%)	1 (3.3%)		1.00-15.45
	G	91 (75.8%)	55 (91.6%)	0.014	3.51
Alleles	A	29 (24.2%)	5 (8.4%)		1.23-12.21

*P-value < 0.05 is significant. INS: Idiopathic Nephrotic Syndrome.

G174C, IL4-C590T, and TNFα-G308A among the INS patients and the controls is represented in Table 3. IL6-G174C, CC genotype and C allele are significantly higher in the INS group compared to the control group (p = 0.003 and 0.009, OR = 4.3 and 2.67 with CI = 1.62 - 11.38 and 1.21 - 6.27 respectively). Also, TNFα-G308A AA genotype and A allele are significantly higher in the INS group than control group (P = 0.045 and 0.014, OR = 3.5 and 3.51, CI = 1-15.45 and 1.23-12.21 respectively). While IL4-C590T shows no significant difference between the 2 groups.

Comparison of the genotypes and allele distribution between the two patient groups (SSNS and SRNS) is represented in Table 4. IL6-G174C is significantly higher in SRNS group than SSNS groups, at the genotypic and allelic levels (p = 0.008 and 0.000, OR = 4.93 and 5.82 respectively). TNFα-G308A, AA genotype is higher in SSNS than SRNS group, though not reaching statistical significant difference (P = 0.279), but the A allele distribution is significantly higher in SSNS group

Table 4. Genotype and allele frequencies of IL6-G174C, IL4-C590T, and TNFα-G308A genes polymorphisms among SSNS & SRNS groups.

SNPs		SSNS (n = 30)	SRNS (n = 30)	*P-value	OR (95% CI)
IL6-G/C	GG	18 (60 %)	7 (23.3%)	Reference	
Genotype frequency	GC	11 (36.7%)	12 (40%)	0.008	4.93
	CC	1 (3.3%)	11 (36.7%)		0.07 - 0.61
Allele frequency	G	49 (81.6%)	26 (43.4%)	0.000	5.82
	C	11 (18.4%)	34 (56.6%)		2.54 - 13.35
IL4-C/T	CC	15 (50%)	18 (60%)	Reference	
Genotype frequency	CT	14 (46.7%)	8 (26.7%)	0.157	0.667
	TT	1 (3.3%)	4 (13.3%)		0.48 - 4.72
Allele frequency	C	44 (73.4%)	44 (73.4%)	1.00	1.00
	T	16 (26.6%)	16 (26.6%)		0.445 - 2.246
TNFα-G/A	GG	17 (56.7%)	22 (73.4%)	Reference	
Genotype frequency	GA	6 (20%)	7 (23.3%)	0.279	2.10
	AA	7 (23.3%)	1 (3.3%)		0.72 - 6.11
Allele frequency	G	40 (66.6%)	51 (85%)	0.032	2.83
	A	20 (33.4%)	9 (15%)		1.08 - 7.81

*$P < 0.05$ is significant, OR: odds ratio, SSNS: steroid sensitive nephrotic syndrome, SRNS: steroid resistant nephrotic syndrome.

compared to SRNS group ($p = 0.032$, OR = 0.2.83, CI = 1.08 to 7.81).

DISCUSSION

Response to Glucocorticoid therapy is the major determinant of prognosis in idiopathic nephrotic syndrome. Despite their broad therapeutic range and effectiveness in remission induction, early markers that would allow optimization of the glucocorticoids dose and duration of the therapy could improve the management of nephrotic patients (Wasilewska et al., 2007).

We studied the association between cytokine genes polymorphisms [IL6-G174C, TNFα-G308A, IL4-C590T and the response to glucocorticoid therapy on 30 patients with SSNS, 30 patients with SRNS compared to 30 control subjects.

On comparing both the INS group and the control group, our results revealed that IL6-G174C CC genotype and C allele are significantly higher among the patients group than the control group (p = 0.003 and 0.009 respectively). Also, on comparing SSNS & SRNS groups IL6-G174C CC and C allele are increased significantly among SRNS group than SSNS group (P=0.008 & 0.000 respectively).

These results are in agreement with the result of 2 studies on Indian population by Tripathi et al. (2008) and Jafar et al. (2011), who reported that IL6-G174C CC genotype and C allele were significantly higher among the patient group (115 SSNS, 35 SRNS) than the control group (569 subjects) with p < 0.001, and the CC genotype only was significantly higher among SRNS group than SSNS group with p = 0.002. In our study, IL6-G174C CC genotype frequency among the control group (6.7%) is in agreement with Helaly et al. (2013), who studied the association of IL6-G174C polymorphism and susceptibility and severity to type 2 diabetes mellitus among Egyptian population on 68 patients and 97 control subjects and revealed that the CC genotype among the control group was 6.1% among the control group (Helaly et al., 2013).

IL6 gene is located at chromosome 7p21 (Sehgal et al., 1986) and consists of 5 exons and 4 introns (Zilberstein et al., 1986). IL6 gene is a polymorphic gene and one of the characterized polymorphisms is a G to C single base change at the promoter site -174 (Olomolaiye et al., 1998). Functional studies have suggested that -174 polymorphism may alter the rate of IL6 gene expression, where the G to C change at position -174 creates a potential binding site for the transcription factor NF-1, which has been shown to be a repressor of IL6 gene expression, so both GG and GC carrying individuals have higher plasma IL6 levels, higher IL6 gene transcriptional activity and higher inducible IL6 response than carriers of CC genotype (Fishman et al., 1998).

IL6 is a multifunctional cytokine that is studied with its genetic variants in immune response, inflammation,

hematopoiesis, and endocrine and nervous system. But the role of IL6-G174C polymorphism in the response to glucocorticoid therapy in nephrotic syndrome is not well studied. In our results, the high frequency of CC genotype in SRNS group than SSNS group may be associated with decreased IL6 expression and decrease the response to glucocorticoids therapy, pointing towards a poor prognosis among those children with SRNS.

As regards to TNF α-G308A polymorphism our results revealed a significant increased association between the INS group and the control group with AA genotype and the A allele being higher in the patient group than the control group (p = 0.045 and 0.002 respectively). While on comparing the two patients groups, only A allele showed a significant higher association with SSNS group than SRNS group (p = 0.032). This is in agreement with Jafar et al. (2011) who found that AA genotype and A allele were significantly higher in the patient group compared to the controls. But contradictory to our results, they found that A allele is higher in SRNS group than in SSNS group, also Kim et al. (2004) found no significant difference between INS patients and control group as regards to TNFα-G308A.

The TNF-α gene is located on the short arm of chromosome 6 within the major histocompatibility complex, where genetic alterations in the TNF-α locus are known to be involved directly in high TNF-α production (Tsukamoto et al., 1998). Several polymorphisms have been identified inside the TNF-α promoter region, among which, a polymorphism that directly affects TNF-α expression is located at nucleotide position −308. A single-base polymorphism within the promoter of the gene for TNF-α results in 2 allelic forms, one in which guanine defines the common allele (G allele) and the other in which guanine is substituted by adenosine forms the rarer allele (A allele) at position −308. TNF-α-A allele is associated with a higher level of TNFA transcript, justified by the greater potency of the promoter region to activate the transcription (Kroeger et al, 1997; Wilson et al, 1997), and the presence of A allele has been found to correlate with enhanced spontaneous or stimulated TNF-α production in both in vitro and in vivo (Mira et al., 1999).

Being one of the immunomodulators, the presence of increased TNF- α-A allele among SSNS group than SRNS group in our study, may play a role in immunomodulation and response to glucocorticoid therapy among the steroid sensitive NS group, thus, it may be a predictor for good response to therapy.

Our results showed no significant difference between the patients group and control group or between the two patients groups as regards to IL4-C590T genotypes or alleles distribution. This is in agreement with Ikeuchi et al who found no significant difference between MCNS patients and control group as regards to IL4-C590T genotypes (Ikeuchi et al., 2009). But, our results are in contrast to Tripathi et al. (2008) and Jafar et al. (2011), who reported that IL4 T allele was significantly higher in INS patients compared to control group, and the TT genotype was significantly higher in SRNS group than SSNS group. Kobayashi et al. (2003) reported that IL4 TT genotype and T allele were significantly lower in MCNS group than the control group. In the present study, the result of IL4-C590T genotype frequencies among the control group (CC = 43.3% and CT= 40%) are in agreement with a study done on Egypt by Alsaid et al. (2013) on the association of IL4-C590T and IL13 genes polymorphisms and susceptibility to type 2 diabetes mellitus on 135 patients and 69 control subjects which revealed CC genotype frequency 43.48% and CT genotype frequency 47.8% among their control group. Also, the results of allele frequencies among our control group (C = 63.3% and T = 36.7%) are in agreement with the control group (C = 36.5% and T = 36.5%) in a study done in Egypt by El-Shabrawi et al. (2011) on IL4-polymorphism in diabetic nephropathy on 100 diabetic patients and 100 control subjects.

The discrepancy between our results and previous reported results in studies on NS may be due to the small sample size included in the study and the different ethnic population.

CONCLUSION

Our findings suggest that polymorphisms at the IL-6 and TNF-α promoter may be useful markers for predicting future response to glucocorticoid therapy and early treatment with immunosuppressive agents in case of SRNS. Further investigations with a larger scale, multicenter studies are necessary to confirm these findings and to elucidate the role of these genes polymorphisms in INS development and course of treatment.

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