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Tumor Necrosis Factor Alpha gene Polymorphisms in Nephrotic Syndrome

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Abstract

Nephrotic syndrome (NS) is one of the most common glomerular diseases characterized by complex pathogenesis with relapses. Many studies mentioned that cytokines act as a potent immunomodulator and as primary candidates for mediating NS progression.

Objective to assess the potential relationships of *TNF-α* SNPs (-238 G/A, and -308 G/A) with the development of NS, and to explore their potential impact on TNF-α serum levels and patient's responses to steroid therapy. This case-control study was conducted in Baghdad city from December 2017 to March 2018. Sixty children (19 female and 41 male) with NS were enrolled in the present study, their age range from 2 to 18 years. They were seeking treatment in the nephrology out patients clinic in Baghdad. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to evaluate different TNF- α gene polymorphism.

Comparison between overall patients and controls revealed a higher level of TNF-α in patients than the controls with a highly significant difference (*P*=0.001). The risk of NS in patients carrying TNF-α- 238 GA genotype, and TNF-α-308 GA or AA genotypes and allele A was significantly increased compared to healthy children. The risk of resistance to steroid therapy was significantly high in NS patients carrying TNF-α-238 GA genotype and A allele also in patients with TNF-α-308 GA, AA genotypes, and A allele.

Total of 238 G/A and -308 G/A TNF- α gene polymorphisms may be risk factors for nonresponsiveness towards steroid therapy among NS children.

Keywords TNF-α, nephrotic syndrome, RFLP

INTRODUCTION

Nephrotic syndrome is a glomerular illness that is characterized by the appearance of proteinuria, hypoalbuminemia, hyperlipidemia, generalized edema and a relapse or remission course (1). Usually, children are more commonly affected, with a decreased quality of life (2).

Nephrotic syndrome pathogenesis is not completely clarified. However, several studies observed that it is related to immune response (3,4). Inflammatory response development and advancement are partly correlated with the proinflammatory (5).

Several cytokine gene polymorphisms, like TNFα, have correlated with various inflammatory diseases as glomerulonephritis and multiple sclerosis (6). Different single nucleotide polymorphisms (SNPs) in the TNF-α gene promoter have been investigated (7,8). These SNPs alter circulating TNF-α level by regulating its production (9,10).

Nephrotic syndrome patients can be classified based on response to glucocorticoids into either steroid-resistant NS or steroid-sensitive NS (glucocorticoids induced remission), about 80% of pediatric NS patients respond to glucocorticoids, while 10-20% being steroid-resistant (11). Variations in steroid response between children are not completely understood and can be assigned to genetic factors (12).

This study aimed to to assess the potential relationships of *TNF-α* SNPs (-238 G/A, and -308 G/A) with the development of NS, and to explore their potential impact on TNF-α serum levels and patient's responses to steroid therapy.

PATIENTS AND METHODS

This case-control study included 60 children suffering from NS (41 males and 19 females, with a mean age of 7.23±3.15 years) and 30 apparently healthy children as control. The control group was matched to cases by age and sex. NS patients were seeking treatment in the nephrology out patients clinic at Al-Imamain Al-Kadhumain Medical City, Central Child teaching Hospital, Al-Karama Teaching Hospital and Children Welfare Teaching Hospital/ Medical City in Baghdad.

Genomic DNA Extraction

DNA was extracted from whole blood samples using a ready kit from Promega Company, USA, according to the manufacturer's instructions**.**

Serum Cytokine Assay

Serum concentrations of TNF- α was measured by using human TNF Alpha PicoKine™ ELISA Kit / Boster Biological Technology –USA Following the manufacturer's instructions.

Genotyping of TNF-α-Gene Polymorphisms

Detection of −238 G/A, and −308 G/A, polymorphisms in the promoter of TNF-α-gene were done using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). Primer sequences (**-238 G/A** F**5'-**AAACAGACCACAGACCTGGTC-**3'** R**5'-**CTCACACTCCCCATCCTCCCGGATC-**3'** with a fragment size 155 bp) (**-308G/A** F**5'-**GAGGCAATAGGTTTTGAGGGCCAT-**3'** R**5'-**GGGACACACAAGCATCAAG-**3'** with a fragment size 147 bp) (13) and PCR conditions for both SNPs were an initial denaturation for 4 min at 94^{o}C. followed by 35 cycles of denaturation for 30 sec at 94^{o}C, annealing for 30 sec (at 63^{o}C for -308, and 61^{o}C for -238) and an extension at 72^{o}C for 45 sec. The final extension was achieved at 72^{o}C for 7 min. PCR products were exposed to digestion by restriction enzymes (Biolab/England). Then, the fragments were electrophoresed in a 2.5% agarose gel (Promega/USA) using 50 bp marker (Promega/US).

Stained with ethidium bromide and visualized under a UV transilluminator.

Data Analysis

All statistical analyses were conducted using Statistical Package for Social Science (SPSS) software version 20. A P< 0.05 (exact two-sided) was accepted as the level of significant. Kruskal Wallis test were used for comparison between the different medians. Binomial variables were expressed as numbers and percentages and analyzed with Chi-square, which was also used to calculate the deviation of different genotypes from Hardy-Weinberg Equilibrium (HWE). Binary logistic regression was used to calculate odds ratio (OR) and the corresponding 95% confidence intervals (CI) in order to assess the association between the different genotypes and alleles of polymorphisms with the risk of NS as well as with the resistant to steroid drugs.

RESULTS

The distribution of different genotypes of the two SNPs was in accordance with Hardy Weinberg equilibrium (HWE). TNF-α 238 G/A polymorphism appeared in only two genotypes: wild-type homozygous (GG) and heterozygous (GA) in both patients and controls. There was no mutant homozygous (AA) genotype neither in patients nor in controls (Figure 1).

Table (1) shows the frequencies of each genotype and alleles of this polymorphism in patients and controls. The frequency of the heterozygous genotype (GA) was higher in NS patients than controls (38.33% versus 16.67%) with a significant difference (OR=3.108, 95%CI=1.043-9.264, *P*=0.042).

At allele level, the minor allele (A allele) was more frequent among patients than controls (19.17% vs 8.33%), however, the difference was not a significant (OR=2.608, 0.939-7.249, *P*=0.066).

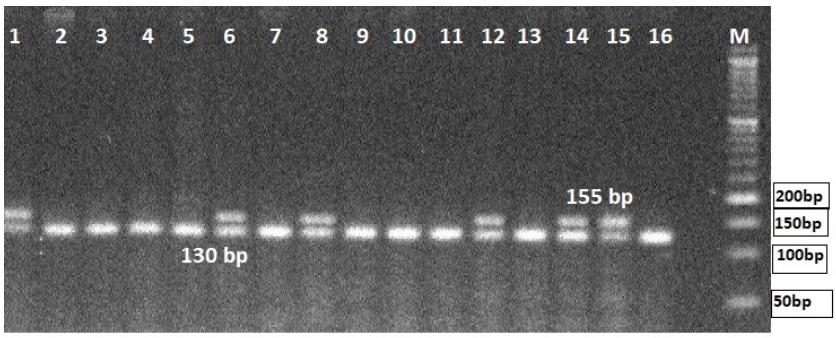

Figure 1: Gel electrophoresis for TNF-α-238 PCR products visualized under U. V light after staining with ethidium bromide. M: 50-1000bpladder; lanes 2,3,4,5,7,9,10,11,13 and 15: wild-type homozygous genotype (GG), lanes 1,6,8,12, 14 and 15 heterozygous genotype (GA).

Table 1: The frequency of different genotypes and alleles of TNF-α-238 polymorphism in nephrotic syndrome patients and controls

TNF-α-238	Patients (60)	Controls (30)	*P*-value	OR(95%CI)
Genotypes				
GG	37(61.67%)	25(83.33%)		1.0 Reference
GA	23(38.33%)	5(16.67%)	0.042	3.108(1.043-9.264)
AA	0(0%)	0(0%)	---	------
HWE	0.066	0.618		
Alleles				
G	97(80.83%)	55(91.67%)	0.066	1.0 Reference
A	23(19.17%)	5(8.33%)		2.608(0.939-7.249)

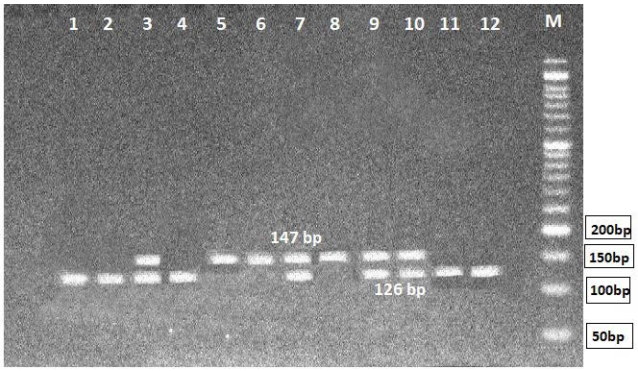

Figure 2: Gel electrophoresis for TNF-α-308 PCR products visualized under U. V light after staining with ethidium bromide. M: 50-1000bpladder; lanes 1,2,4,11 and 12: wild-type homozygous genotype (GG), lanes 3,7,9 and 10: heterozygous genotype (GA), lanes 5 and 6: mutant homozygous genotype (AA).

Table 2: The frequency of different genotypes and alleles of TNF-α-308 polymorphism in nephrotic syndrome patients and controls.

TNF-α - 308	Patients (60)	Controls (30)	*P*-value	OR(95%CI)
TNF-a				
Genotypes				
GG	24(40%)	20(66.67%)	0.051	1.0 Reference
GA	23(38.33%)	8(26.67%)	0.087	2.396(0.882-6.51)
AA	13(21.67%)	2(6.67%)	0.039	5.417(1.091-26.899)
HWE	0.109	0.361		
Alleles				
G	71(59.17%)	48(80%)	0.006	1.0 Reference
A	49(40.83%)	12(20%)		2.761(1.331-4.727)

Table 3: The frequency of different genotypes and alleles of TNF-α-238 polymorphism in steroid sensitive and steroid-resistant patients.

TNF-α-238	Steroid sensitive patients (30)	Steroid resistant patients (30)	*P*-value	OR(95%CI)
Genotypes				
GG	22(73.33%)	15(50%)		1.0 Reference
GA	8(26.67%)	15(50%)	0.066	2.75(0.934-8.1)
AA	0(0%)	0(0%)	---	------
Alleles				
G	52(86.67%)	45(75%)	0.109	1.0 Reference
A	8(13.33%)	15(25%)		2.167(0.841-5.582)

Table 4: The frequency of different genotypes and alleles of TNF-α-308 polymorphism in steroid sensitive and steroid-resistant patients.

TNF-α-308	Steroid sensitive patients (30)	Steroid resistant patients (30)	*P*-value	OR(95%CI)
Genotypes				
GG	15(50%)	9(30%)	0.189	1.0 Reference
GA	11(36.67%)	12(40%)	0.314	1.818(0.568-5.817)
AA	4(13.33%)	9(30%)	0.072	3.75(0.89-15.808)
Alleles				
G	41(68.33%)	30(50%)	0.042	1.0 Reference
A	19(31.67%)	30(50%)		2.158(1.027-4.536)

TNF-α 308 G/A polymorphism had three genotypes (GG, GA, and AA) in patients and controls as illustrated in figure (2).

Table (2) shows the frequency of the GG, GA and AA genotypes of TNF-α-308was 40%, 38.33%,and 21.67% respectively in patients compared to 66.67%, 26.67%,and 6.67% respectively in controls. Logistic regression analysis revealed a significant difference in the frequency of mutant homozygous genotype (AA) between the two groups (OR= 5.417, 95%CI=1.091-26.899, *P*=0.039). This difference was more prominent at allele level where the frequency of a mutant allele (A allele) in patients was more than twice of that in control with a highly significant difference (OR=2.761, 95%CI=1.331-4.727, *P*=0.006).

Although the heterozygous genotype (GA) accounted for exactly half total genotypes among steroid-resistant patients compared to only 26.67% among steroid-sensitive patients, this difference was not enough to reach a statistically significant (OR= 2.75, 95%CI= 0.934-8.1, *P*=0.066). Likewise, the mutant allele was more frequent among steroid resistant than steroid sensitive patient (25% vs 13.3%); however the difference was not a significant (OR= 2.167, 95%CI=0.841-5.582, *P*=0.109) as shown in the table (3).

No significant differences in the distribution of different genotypes of TNF-α-308 polymorphism between steroid sensitive and steroid resistance patients although the mutant homozygous genotype (AA) was more frequent in steroid resistance (30%) than steroid sensitive (13.33%) as shown in the table (4). On the other hand, the frequency of allele A in steroid sensitive and steroid-resistant patients were 50% and 31.67% respectively with a significant difference (OR= 2.158,95%CI=1.027-4.536, *P*=0.042).

Table (5) shows the influence of TNF-α-238 polymorphism on the serum levels of TNF-α in patients and controls. Interestingly, the median level of TNF-α in NS patients carrying genotype GA was significantly higher than those patients carrying the GG genotype (*P*=0.03). Stratification of patients into steroid sensitive and steroid-resistant reduced the number of samples and abolished the significant difference.

Table 5: The impact of TNF-α-238 on serum levels of TNF-α measured by pg/ml (median, range)

Status	GG	GA	*P*-value
Controls (30)	2.5 (0-21)	4 (0-47)	0.192
NS patients (60)	8 (0-99)	17 (0-111)	0.036
Steroid sensitive patients (30)	8.5 (0-24)	11.5 (0-99)	0.26
Steroid resistant patients (30)	12 (0-71)	18 (0-88)	0.068

Table 6: The impact of TNF-α-308 on serum levels of TNF-α measured by pg/ml (median, range)

Status	GG	GA	AA
Controls (30)	1.5(0-26)[a]	3 (0-28)[b]	5(0-47)[c]
NS patients (60)	13(0-99)[a]	9 (1-88)[a]	15 (0-85)[b]
Steroid sensitive patients (30)	7(0-71)[a]	12(0-85)[a]	11 (1-88)[b]
Steroid resistant patients (30)	13.5 (0-24)[a]	14 (1-111)[b]	26(2-99)[c]

Different small letters indicate significant differences

TNF-α-308

High levels of TNF-α were almost always associated with the mutant genotype (AA) of this polymorphism regardless of the subject status. In controls, median levels of TNF-α in GG, GA and AA genotypes were 1.5 pg/ml (range 0-26), 3 pg/ml (range 0-28) and 5 pg/ml (range 0-47) respectively with significant differences between the three genotypes. Likewise, AA carrier patients as a whole and as steroid sensitive showed the significantly higher median level of this cytokine compared with either GG carriers and GA carriers, with no significant difference between the last two genotypes. In steroid-resistant patients, the three genotypes differed significantly from each other, with a higher level of the cytokine associated with AA genotype followed by GA and finally GG (Table 6).

DISCUSSION

TheCurrent study revealed a considerably higher risk of the NS among people who have TNF-α-308 AA or GA genotypes and A allele than people carrying GG genotype and G allele. Comparable to present result are Manal *et al.,*Yosuf *et al.,* and Madani *et al.*(13,14) in Egypt also Jafar *et al;* in India (3) who registered a significantly higher AA genotype and A allele. In contrast, Kim *et al.* stated that there was no relation of TNF-α-308 polymorphism with the risk of NS (15).

Regarding the comparison between steroid sensitive nephrotic syndrome (SSNS) and steroid resistant nephrotic syndrome (SRNS), results revealed that the frequencies of TNF-α -308 GA, AA genotypes were more frequent in SRNS than SSNS children. The risk of resistance to steroids was higher among children carrying GA or AA genotypes than children carrying GG genotype and this similar to Youssef *et al,* (13) and Jafar *et al,* (3) findings.

These results illustrate that A allele may be responsible for the risk and the responsiveness to treatment, it was highly expressed in SRNS than SSNS.

In the case of the TNF promoter SNP, −308 G/A, some reports concluded that the −308 A allele yielded an increase in transcription in reporter assays, so the presence of A allele was associated with higher levels of TNF expression (16).

Concerning to TNF-α-238 SNP, the GG genotype was dominant in controls and in NS patients, whereas the AA genotype was missing in both groups. Comparable to this data, Yousif *et al* and Mousa *et al.* registered missing of TNF-α-238 AA genotype in NS patients and control group (13, 17). On the other hand, the current study showed, the remarkably higher risk of NS was in people having GA genotype and A allele than other carrying GG genotype and G allele. Interestingly, TNF-α-238 SNP was correlated with an increase in susceptibility to inflammatory and immunological diseases (18).

In addition, the risk of resistance to steroid therapy was higher in children with NS carrying GA genotype compared to children carrying GG genotype but statistically not significant and this is may be due to the small sample size because it was divided to SRNS and SSNS.

The current study observed that the level of serum TNF-α among nephrotic syndrome cases was higher than the control group. Also, in children with the SSNS, TNF-α level decreased after corticosteroid therapy. This finding suggests that disease activity, at least in some NS children, may be correlated with TNF-α serum levels. This assumption is further supported by the lack of a reduction in serum TNF-α levels in the patients with SRNS.

SSNS children showed a low level of serum TNF-α compared with SRNS and this may be due to treatment with prednisolone because prednisolone can inhibit leukocyte infiltration at the site of inflammation, interfere with mediators of inflammatory response, and suppress humoral immune responses. Also, prednisolone reduces inflammatory reaction by limiting the capillary dilatation and permeability of the vascular structures. These compounds restrict the accumulation of polymorphonuclear leukocytes and macrophages and reduce the release of vasoactive kinins (19)

Regarding -308 G/A, the present results showed that patients with AA and GA genotype showed significantly higher TNF-α level than patients carrying GG genotype. As well as A allele is associated with a high level of serum TNF-α.

Previous studies observed that 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 (20,21) 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 (22).

For -238 G/A , the result showed that patient carrying GA genotype has a higher TNF-α than patient carrying GG genotype. This may be explained by the strong connection

of A to the RNA polymerase on the promoter region of TNF- α leading to increasing TNF-α transcription and production. Also, the G>A transition polymorphism at 308 positions 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 (21). TNF-α activates NF-κB and angiotensinogen (3,23) hence steroid-induced immunosuppression is critically dependent upon inhibition of NF-κB. Elevation of TNF-α has been found in the plasma and urine of patients with NS (24). The transcriptional and post-transcriptional alterations of NF kappa B/ I-kappa B alpha in nephrotic syndrome (25) can be a potential pathway for TNF-α action in determining steroid responsiveness.

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