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Tumor necrosis factor alpha gene polymorphisms and haplotypes in Egyptian children with nephrotic syndrome

Doaa M. Youssef[a], Amal S. El-Shal[b,*], Samia Hussein[b], Khaled Salah[a], Abd El Rahman E. Ahmed[a]

[a] Pediatrics Department, Faculty of Medicine, Zagazig University, Egypt
[b] Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University, Egypt

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ABSTRACT

Background: Nephrotic syndrome (NS) characterized by complex pathogenesis and clinical course with relapses; and needs novel breakthroughs for decades. Polymorphisms of cytokines genes including tumor necrosis factor alpha (*TNF-α*) may influence susceptibility to NS as well as different patients' steroid responses. In the current study, we demonstrated the potential roles of *TNF-α* promoter gene polymorphisms [−238, −308, −863] and haplotypes in susceptibility to childhood NS. Also, elucidating their possible influence on patients' steroid response and serum TNF-α level.

Methods: This case-control study included 150 children suffering from NS and 150 healthy children. Polymerase chain reaction- restriction-fragment length polymorphism (PCR-RFLP) was performed to evaluate different TNF-α gene polymorphism. TNF-α serum levels were assessed by ELISA.

Results: Serum TNF-α levels were significantly higher in NS patients than in controls and in steroid resistant NS (SRNS) than in steroid sensitive NS (SSNS) ($P < 0.001$ for each). 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. While no significant association was detected between *TNF-α-863* and NS. The risk of resistance to steroid therapy was significantly high in NS carrying *TNF-α-238* GA genotype and A allele, *TNF-α-308*, AA genotypes and A allele, and *TNF-α*-863 CA, AA genotypes and A allele. The *TNF-α* GCG (−308/−863/−238) haplotype has protective roles against NS and steroid resistance. However, the risk of NS was significantly high in *TNF-α* AAG and AAA haplotype's carriers compared to healthy children. Additionally the risk of steroid resistance was significantly high in *TNF-α* AAA haplotype's NS carrier (OR (95%CI): 2.2 (1.19–4.36), $P = 0.01$). Moreover, we found significant higher serum TNF-α levels NS patients including SSNS and SRNS carrying mutant allele *TNF-α*-238 GA genotype, −308 GA and AA and −863 CA and AA wild genotype's carriers than in those GG, GG and CC respectively. Interstingely, TNF-α levels were significantly higher in healthy children carrying *TNF-α*(−308/−863/−238) [AAG and AAA haplotypes], NS cases carrying [ACA, AAG, AAA haplotypes], and in SSNS carrying [ACA and AAA haplotypes] than in those carrying GCG, haplotype of wild alleles.

Conclusion: This study reported, for the first time, that *TNF-α* promoter gene polymorphisms and/or haplotypes are risk factors of NS and resistance to steroid among Egyptian children.

1. Introduction

Nephrotic syndrome (NS) is a common problem in children with a prevalence of about 1–3 per 100,000. It is manifested by heavy proteinuria, hypoalbuminemia, hyperlipidemia, and edema [1]. The most frequent form is Idiopathic NS (INS) and the most common histopathological type in INS is minimal change NS (MCNS) [2]. In general, it is of good prognosis but relapses are very common which in turn causes several complications [3].

Approximately 85–90% of INS children are steroid responsive with complete remission, while, 10–15% are partial or steroid resistant NS (SRNS) [4]. Differences in steroid response among children are not fully understood and can be attributed to genetic factors [5]. Notably, an improvement on steroids indicates a favorable prognosis [6].

The pathogenesis of INS is not fully clarified. However, some evidence showed that it is linked to immune response [7–9]. The development and the advancement of the inflammatory response is partially related to tumor necrosis factor alpha (TNF-α) as a proinflammatory cytokine [10]. It may provoke the expression of other proinflammatory cytokines [11–13]. Different single nucleotide polymorphisms (SNPs) in

* Corresponding author.
E-mail address: amalelshal@gmail.com (A.S. El-Shal).

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Table 1
Primers sequences, restriction enzymes and product and fragement sizes for genotyping of *TNF-α* gene polymorphism.

SNP Locus	Primers sequences	PCR conditions	Product size	Restriction enzyme	Fragment size
-238 G/A	F:5'-AAACAGACCACAGACCTGGTC-3' R:5'-CTCACACTCCCCATCCTCCCGGATC-3'	94°C for 4 min 94°C for 30 sec 61°C for 30 sec 72°C for 45 sec } 35 cycles 72°C for 7 min	155 bp	BamH I	G allele: 130+25 bp A allele: not digested
-308 G/A	F:5'- GAGGCAATAGGTTTTGAGGGCCAT-3' R:5'- GGGACACACAAGCATCAAG -3'	94°C for 4 min 94°C for 30 sec 63°C for 30 sec 72°C for 45 sec } 35 cycles 72°C for 7 min	147 bp	NcoI	G allele: 126+21 bp A allele: not digested
-863 C/A	F:5'- GGCTCTGAGGAATGGGTTAC -3' R:5'- CCTCTACATGGCCCTGTCTAC -3'	94°C for 4 min 94°C for 45 sec 53°C for 45 sec 72°C for 60 sec } 35 cycles 72°C for 6 min	126 bp	Tai I	C allele: not digested A allele: 105+21 bp

SNP, single nucleotide polymorphisms

the *TNF-α* gene promoter have been investigated [14–17]. These SNPs alter circulating TNF-α level by regulating its production [18,19].

Therefore, we tried to assess the potential relationships of *TNF-α* SNPs (238 G/A, −308 G/A, and −863 C/A) and haplotypes with risk of NS, and to explore their potential impact on TNF-α serum levels and patient's responses to steroid therapy.

2. Subject and methods

This case-control study included 150 children suffering from NS (76 males and 74 females, with a mean age of 4.4 ± 1.8 years) and 150 healthy children (71 males and 79 females, with a mean age of 4.7 ± 1.9 years). The control group was matched to cases by age, sex with no history of steroid therapy. The patients were recruited from Nephrology unit, Paediatric Department, Zagazig University. All study subjects were from the Delta region of Egypt. Clinical findings of NS cases were obtained from hospital records. The study was performed after approval by the Institute Review Board of Faculty of Medicine, Zagazig University, and children parents' assigned written consent.

NS was diagnosed by massive proteinuria of ≥40 mg/h/m^2 and hypoalbuminemia of ≤2.5 g/dl with unknown causes [21]. According to their initial response to steroid therapy, the NS cases were classified into steroid sensitive (SS) or steroid resistant (SR). The children with NS were deemed SS if they fulfilled the following criteria: disappearance of proteinuria for 3 consecutive days, or had a urine protein/creatinine level of < 0.2 within the first 4 weeks of full dose prednisolone therapy. On the other hand, children with SRNS were diagnosed by absence of remission after a full dose of oral corticosteroid therapy for 1 month followed by 3 pulses of intravenous methylprednisolone [22].

Remission was diagnosed by the disappearance of albuminuria for at least 3 consecutive days. Additionally, frequent relapse was diagnosed by more than two relapses within the initial 6 months after the presentation or more than four relapses per year during follow-up [23]. Also, steroid dependant NS (SDNS) was diagnosed by at least two relapses during alternate-day treatment with prednisone or within 2 weeks after cessation [24]. Of those with NS who were initially SS, 56 (37.4%) children had infrequent relapses, 44 (29.3%) children had frequent relapses and 50 (33.3%) children were SR. Mesangioproliferative NS (MCNS) pattern was found in 48 (32%) children and 63 (42%) children were found to have early focal segmental glomerulosclerosis pattern. While renal biopsy was not done in 39 NS patients (26%) because their parents had not given consent for kidney biopsy; these children were mainly non-frequent relapser NS.

2.1. Sampling of blood

One ml of venous blood was taken from all children into EDTA-containing tubes for ELISA and DNA extraction.

2.2. Sampling of urine

Sterilized containers were used for 24 h urine samples collection and were used to estimate 24 h urinary protein.

2.3. Biochemical assessment

A 24 h urinary protein, blood creatinine and urea levels were assessed by commercially available kit (Spinreact, Girona, Spain). TNF-α levels were determined with human TNF-α Quantikine ELISA kit (R & D Systems, Minneapolis, MN, USA).

2.4. Genomic DNA extraction

QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) was used for extraction of genomic DNA from peripheral blood following the instructions of the manufacturer.

2.5. Genotyping of TNF-α-gene polymorphisms

Detection of −238 G/A (rs361525), −308 G/A (rs1800629), and −863 C/A (rs1800630) polymorphisms in the promoter of *TNF-α*-gene were done using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) according to Zuo et al. [19] for −238 G/A and −308 G/A and Skoog et al. for −863 [25]. Primer sequences and PCR conditions for each SNP were listed in Table 1.

PCR amplification was done using thermal cycler, PERKIN ELMER (Norwalk, CT, USA). PCR reaction mixture of total volume (25 µl) including 100 ng genomic DNA (7 µl), 1.0 µM (0.5 µl each primer) of each primer (Promega, Madison, USA), 4 µl DdH_2O, and 12.5 µl of Taq PCR master mix 2X (Qiagen GmbH, Hilden, Germany). PCR products were exposed to digestion by restriction enzymes (Fermentas–Euromedex, France). Then, the fragments were electrophoresed in a 3.5% agarose gel (Serva, Spain) using 50 bp marker (Fermentas, Germany) stained with ethidium bromide and visualized under a UV transilluminator.

2.6. Statistical analysis

Statistical analyses were done using the Statistical Package for the