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TNF-α (-308 G→A) Polymorphism and the Risk of Progression to End Stage Renal Disease in Nephropathy Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author IH performed the study and wrote the manuscript. Author SRM designed the study, provided clinical assistance and did the data analyses. Authors PAM and SAM provided procedural assistance and helped in literature survey. Author NAN performed proof reading and statistics. Author BAG designed and conceived the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This hospital based case-control study sought to analyze the association between the promoter region polymorphism in TNF-α and the risks of developing end stage renal disease in nephropathy subjects.
Methodology: 222 documented cases of end stage renal disease (Males=148, Females=74) subjects and 250 healthy controls (Males=130, Females=120) were included in the study. Among

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222 cases, 126 subjects had hypertensive nephropathy and 96 had glomerulonephritis as contributing factors of ESRD. Clinical and demographic data was collected from each case. TNF-α (-308 G→A) promoter polymorphism was analyzed by selective amplification by polymerase chain reaction and subsequent digestion by *NcoI* restriction enzyme. Genotypic and allelic frequencies were compared to controls using Chi-square and Odds ratio analysis. Clinical parameters were compared across genotypes using logistic regression. The probability values were adjusted for age and gender.

Results: Mean age of cases and controls was 47.78 and 46.83 years respectively. Genotypes for TNF-α -308 followed Hardy Weinberg equilibrium (*P*=0.17). The frequency of homozygous wild, heterozygous and homozygous rare genotypes in cases and controls was 62.6%, 31.08%, 6.3% and 74%, 23.6%, 2.4% respectively (*P*=0.019). Comparison of genotypes between cases and controls showed an association of AA genotype with ESRD (*P*=0.02, OR=3.1, 95%CI=1.1-6.2). The AA genotype was significantly associated with lower age in cases (*P*=0.008) as well as lower serum protein (*P*=0.03) and calcium levels (*P*=0.01).

Conclusion: TNF-α (-308 G→A) promoter polymorphism is associated with nephropathy and the carriers of AA genotype exhibit an increased risk towards rapid progression of ESRD.

Keywords: TNF-α; ESRD; nephropathy; glomerulonephritis.

1. INTRODUCTION

End stage renal disease (ESRD) is regarded as a global public health issue with relatively unchanged magnitude and mortality despite growing technological advancement and progress in renal replacement therapy [1,2]. In ESRD patients, dysfunctional excretion by kidneys results in anomalous immune functions and building up of uremic toxins [3,4]. Overproduction of pro-inflammatory cytokines as a result of altered expression of immune cells has been correlated with renal injuries [5]. Several studies demonstrate an increased turnover of various cytokines in ESRD patients. The rate of cytokine turnover differs individually and maybe related to genetic susceptibility [6-9]. Allelic polymorphisms in the promoter region of cytokines are associated with the inter-individual capacities to synthesize and secrete varying amounts of cytokines [5,10]. Tumor necrosis factor-α (TNF-α) is an important pro-inflammatory cytokine produced early in the inflammatory process that causes up regulation of other cytokines as well as TNF-α itself. The role of TNF-α in modulating the progression to ESRD has been suggested by several studies [7,9,11-13]. Over-expression is in part determined by the transcriptional modifications due to genetic variations. In TNF-α gene, a single nucleotide polymorphism (SNP) located at position -308 of its promoter region is associated with varying production of TNF [14]. Data from various studies suggest that the TNF-α-308 (G→A) polymorphism modifies the TNF production and is associated with over-production of TNF in experimental studies [15,16]. Very few studies have confirmed this association in ESRD patients [17,18]. Thus the present study was undertaken to assess the relationship between TNF-α-308 (G→A) SNP and ESRD.

2. MATERIALS AND METHODS

2.1 Study Population

All subjects enrolled in the study belonged to ethnic population of Kashmir valley; a geographically distinct region located in north India. The study protocol was in conformity with the 2013 Declaration of Helsinki and was approved by the institutional ethics review board. Written informed consent was obtained from subjects willing to participate in the study. 222 (148 Male, 74 Females) documented cases of nephropathy related ESRD as defined by The National Kidney Foundation–Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines [19] and 250 healthy controls (130 Male, 120 Females) were included in the study. 126 cases had hypertensive nephropathy and 96 [MGN (n=36), RPGN (n=30, polycystic (n=6), IgA (n=18), idiopathic (n=6)] had glomerulonephritis as a contributing factor of ESRD. Demographic and biochemical parameters were determined from the fasting venous blood sample of each patient by an automatic analyzer. eGFR was determined by CKD Epidemiology Collaboration (CKD-EPI) Creatinine equation [20].

2.2 Isolation of Genomic DNA

DNA was isolated from whole blood using Quick-gDNA™ Blood Mini Prep kit from Zymo

Research as per the instruction manual. The purity and concentration of isolated DNA was determined spectrophotometrically using Nanodrop. Integrity of isolated DNA was checked by electrophoresis using 0.8% agarose gel.

2.3 Genotyping TNF-α -308 SNP

TNF-α (-308 G→A) SNP was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as reported earlier [3]. A 107bp promoter region of TNF-α encompassing the SNP was amplified using forward 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and reverse 5'-TCC TCC CTG CTC CGA TTC CG-3' primer pair [18]. Amplification was carried out in in the ABI Thermal Cycler (Applied Biosystems, Foster City, USA) in a final volume of 25 µl using 100ng of DNA, 5 µM of each primer, and 1 U of Taq DNA polymerase supplied with buffer enzyme 1X (2.5 µl), 2.5 mM of MgCl2, and 1.5 mM of dNTP mix (Invitrogen Life Technologies, Carlsbad, USA).

PCR protocol was set as per following cyclic conditions: initial denaturation at 95℃ for 5 min, 30 cycles of denaturation at 95℃ for 45 s, annealing at 61℃ for 30 s, extension at 72℃ for 1 min, followed by a final extension at 72℃ for 7 min. The amplified product was digested using 1U of restriction enzyme *NcoI* (Fermentas, USA) by incubating the reaction mixture overnight at 37℃. Restriction products (homozygous GG=97+20, homozygous AA=117 and heterozygous GA=117+97+20) were analyzed on 3% agarose gel and photographed on a gel documentation system (Supplementary Fig. 1). The genotypic results were further validated by Direct Sequencing of 10% of PCR products.

2.4 Statistical Analyses

Data was managed using Microsoft Excel and SPSS 20.0 statistical package (SPSS, Chicago, IL, USA). Demographic and clinical data were presented as percentage, mean value and standard deviation (SD). Hardy-Weinberg Equilibrium (HWE) and genotype/allelic proportions were tested using the Chi square (χ^2) and Fischer exact test. Association between genotypes and disease was measured using Odds ratios (OR) and 95% confidence intervals (Vassar Stats software). For non-parametric data analysis, Mann-Whitney U test was performed. Results were considered significant at $P < 0.05$.

3. RESULTS

3.1 Clinical and Demographic Results of Cases

472 subjects comprising of 222 cases and 250 controls were included in this prospective case-control study. Mean age of cases and controls was 47.78 and 46.83 years respectively. Majority of cases were males (148/222) Demographic and clinical data of cases is shown in Table 1.

Table 1. Demographic and clinical parameters of cases

Parameter	All cases (N=222)	Hypertensive nephropathy (N=126)	Glomerulonephritis (N=96)
Demographic			
Age (years)	47.8±14.9	58.3±6.5	33.81±11.0
Gender (male %)	67	71	62
Clinical			
Systolic BP (mmHg)	147±15.3	154.4±18.8	137.2±16.4
Diastolic BP (mmHg)	91.8±9.2	96.1±6.5	86.4±9.3
Smoking history (%)	37	66	6
Urea (mg/dl)	132.3±59.2	132.3±64.9	134.4±51
BUN (mg/dl)	63.3±29.8	66.15±34.4	65.27±29.2
Creatinine (mg/dl)	6.49±3.26	6.2±2.8	6.8±3.7
Serum proteins (g/dl)	6.31±0.84	6.6±0.7	5.88±0.6
Calcium (mg/dl)	7.13±1.5	7.40±0.8	6.7±2.0
Phosphorus (mg/dl)	5.18±2.14	5.13±2.20	5.4±2.1
eGFR (ml/min/1.73 m^2)	11.38±6.6	10.85±6.4	11.5±6.03

Data expressed in percentage and Mean±SD

3.2 Genotypic and Allelic Frequency Distribution of TNF-A -308 Polymorphism

The frequency of genotypes of TNF-α (-308 G→A) for both cases and controls were in Hardy-Weinberg Equilibrium [*P*=0.6 (controls), *P*=0.17 (cases)]. The frequency of homozygous wild, heterozygous and homozygous rare genotypes in cases and controls is shown in the histogram (Fig. 1).

3.3 Association between the GG, GA and AA Genotypes and Clinical Variables in Cases

We performed analysis of variance between clinical parameters across the GG, GA and AA genotypes and observed a difference between AA genotype and lower age (GG=48.29±12.43, GA=48.57±18.6, AA=38.86± 15.29, *P*=0.008) (Table 3). We also observed a significant difference for low serum proteins

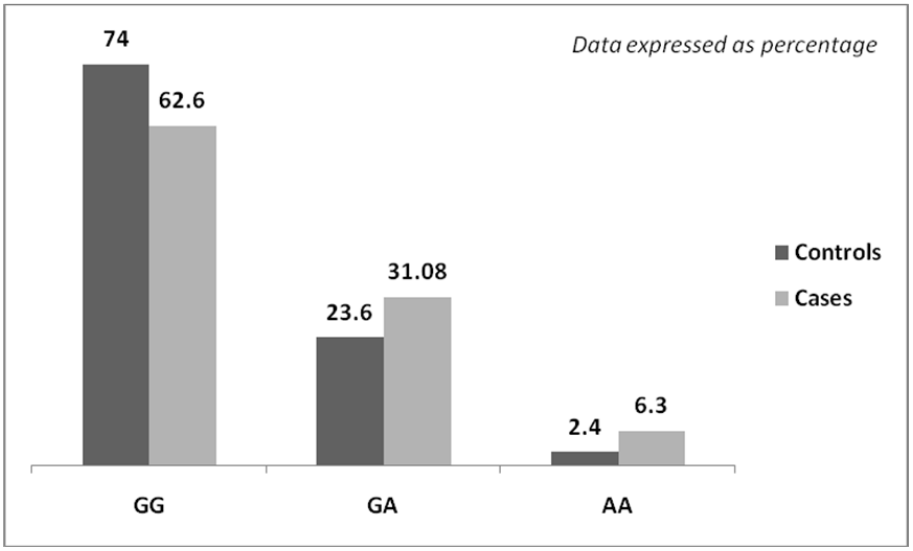

Fig. 1. Histogram showing genotypic frequencies of TNF-α (-308 G↔A) SNP

Table 2. Genotype frequencies and association of TNF-α (-308 G↔A) SNP with study subjects

Model	Genotype/Allele	Cases (N=222)	Controls (N=250)	OR (95% CI)	*P* value
Codominant	GG	139	185	1	Referent
	GA	69	59	1.5 (1.03-2.34)	0.03
	AA	14	6	3.1 (1.1-6.2)	0.02
	G	347	429	1	Referent
	A	97	71	1.6 (1.2-2.3)	0.002

Interaction P value adjusted for age and gender=0.019

Table 3. Association of TNF-α (-308 G↔A) genotypes with different variables among cases

Variable	GG	GA	AA	*P*
Age (years)	48.29±12.43	48.57±18.6	38.86±15.29	.008
Systolic BP (mmHg)	147.5±14.04	146.4±17.2	144.2±17.85	.41
Diastolic BP (mmHg)	92.16±9.06	91.74±9.6	90.1±9.7	.39
Urea (mg/dl)	127.43±64.5	140.7±45.8	139.9±61.7	.48
BUN (mg/dl)	59.55±30.14	65.77±21.42	65.39±28.84	.48
Creatinine (mg/dl)	6.53±3.5	6.22±2.64	7.46±3.2	.34
Serum proteins (g/dl)	7.75±0.77	6.40±0.85	6.20±0.82	.03
Calcium (mg/dl)	7.35±1.23	7.01±0.85	6.64±1.02	.01
Phosphorus (mg/dl)	5.01±2.03	5.67±2.42	4.5±1.28	.04
eGFR (ml/min/1.73 m^2)	11.24±6.5	11.9±6.7	9.93±6.8	.47

(GG=7.75±0.77, GA=6.40±0.85, AA=6.20±0.82, *P*=0.03) and calcium (GG=7.35±1.23, GA=7.01±0.85, AA=6.64±1.02, *P*=0.01) levels. Other clinical parameters did not show any statistically significant association upon comparison.

4. DISCUSSION

TNF-α is a key inflammatory cytokine that is synthesized and released by blood mononuclear cells [21]. TNF-α is also expressed and released by various renal cells [22]. TNF-α secretion is also linked to the stimulation of other pro-inflammatory cytokines that play a crucial role in modulating renal function [23,24]. Alterations in TNF-α gene expression has been associated with several autoimmune and infectious diseases and may play a role in the pathogenesis of ESRD [16,25]. In the present study we analyzed the association between TNF- α (-308 G→A) SNP and nephropathy related ESRD. The frequency of AA genotype was higher in patients as compared to control subjects. We observed a positive association between the carriers of AA genotype and the disease (*P*=0.02). Though the studies investigating this SNP as a predisposing risk factor for nephropathy/ESRD is conflicting due to inter-individual differences and phenotypic heterogeneity [17,18,26-28], our data however is in conformity with the results of several studies [17,18]. The homozygous rare AA genotype of TNF-α -308 SNP is correlated with increased TNF-α production that in turn is associated with a 25-folds increased risk for ESRD (OR=25.02, *P*<0.001) [18]. The data from our study affirms this risk in a relatively larger sample size with better study power. While correlating the clinical and demographic parameters with the genotypes, we observed a significant association between AA genotype and lower age in cases (*P*=0.008). TNF-α AA genotype is associated with an increased expression of TNF-α levels, carriers of AA genotype may experience an exacerbated inflammatory response. This association relates to either early onset of symptoms or rapid progression towards ESRD. Age has been reported as an important effect modifier in renal disease [29]. Studies show different prognostic implications in renal diseases with respect to age [30]. The significance of age difference in ESRD reflects various phenomena. Lower incidence of ESRD among older patients is likely due to the greater competing risk for death as a result of a variety of other age-related coexisting co-morbidities [29]. Other explanation could be that older patients are long-term survivors and thus have non-progressive or slowly progressive disease than younger patients. In older patients, age-related co-morbidities tend to predict global outcomes like mortality whereas in younger patients specific renal outcomes can be predicted [30]. The association between TNF-α-308 AA genotype was also significantly associated with lower serum protein and calcium levels in our study. This association can be explained by increased urinary loss of protein (*P*=0.03) and calcium (*P*=0.01) in such cases. Hypocalcemia has been observed more often in ESRD and is quite uncommon in stages preceding ESRD [31]. The increased proteinuria and hypocalcemia in patients with A/A genotype implicate peritoneal membrane dysfunction which is found in ESRD.

The limitations of the study include low sample size, disease heterogeneity and effect of unmeasured confounding factors.

5. CONCLUSION

Thus, the study demonstrates that the variation in the promoter region of TNF-α gene resulting in G to A transition is associated with nephropathy patients who progressed to ESRD. As a result of inter-individual genetic variations, TNF-α polymorphism maybe an important risk factor or therapeutic target in patients prior to the development of ESRD. Future considerations include prospective cohort studies to determine whether incident ESRD among nephropathy patients is higher among patients with TNF-α polymorphism.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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