

Original Research Article

Association between TNF- α (-308 G $\leftrightarrow$ A) polymorphism and Nephropathy (End Stage Renal Disease)

ABSTRACT

Aims: the 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.

Study design: Prospective, Hospital based case-control study.

Place and Duration of Study: Department of Biochemistry, University of Kashmir, Srinagar and Department of Endocrinology and Metabolism, SKIMS, Srinagar, between 2013-2015.

Methodology: 222 (148 Male, 74 Females) documented nephropathy subjects with end stage renal disease and 250 healthy controls (130 Male, 120 Females). Clinical and demographic data was collected from each case. TNF- α (-308 G $\leftrightarrow$ A) promoter polymorphism by analyzed by selective amplification by polymerase chain reaction and subsequent digestion by *NcoI* restriction enzyme. Genotypic and allelic distribution was performed by Chi-square and Odds ratio analysis. Clinical parameters were compared across genotypes by logistics. The probability values were adjusted for age and gender.

Results: Mean age of cases and controls was 47.78 and 46.83 years respectively. 67% of cases were males. Genotypes for TNF- α -308 followed Hardy Weinberg equilibrium ($P=.17$). 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. Comparison of genotypes between cases and controls showed a positive association of AA genotype with nephropathy related ESRD ($P=0.02$, OR=3.1, 95%CI=1.1-6.2). The AA genotype was significantly associated with lower age in cases ($P=.008$) as well as lower serum protein ($P=.03$) and calcium levels ($P=.01$).

Conclusion: TNF- α (-308 G $\leftrightarrow$ 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, polymorphism

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 Acute kidney injury [5].

Several studies demonstrate an increased turnover of various cytokines in ESRD patients with a significantly apt role of due to certain genetics in its susceptibility [6-9]. The existence of a Allelic polymorphism

especially in the DNA promoter region of cytokines is associated with the inter-individual capacities to

synthesize and with secreting 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. [9][K1] the role of TNF- α in modulating the progression

to ESRD has been suggested implicated by several studies [7, 8, 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 [K2] is associated with

varying production of TNF [14]. Data from various studies suggest that the -308 (G $\leftrightarrow$ A) polymorphism modifies the TNF production and the association of A allele with over-production of TNF is also observed in vitro and in vivo [14, 15]. Very few studies have confirmed this association in ESRD patients [15, 28]. Thus the present study was undertaken to assess the relationship between TNF- α -308

(G $\leftrightarrow$ A) SNP and the risk of developing ESRD.

2. MATERIAL 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 [16] and 250 healthy controls (130 Male, 120 Females) were included in the study. Routine biochemical parameters [K3] 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 [17].

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 [K4]. 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 $\leftrightarrow$ 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 the ABI Thermal Cycler (Applied Biosystems, Foster City, USA). Amplification was carried out in a final volume of 25 μ l using 100ng of DNA, 5 mM of each primer, and 1 U of Taq DNA polymerase supplied with buffer enzyme 1X (2.5 μ l), 3.5 mM of MgCl₂, and 5 mM of each dNTP (Invitrogen Life Technologies, Carlsbad, USA).

PCR protocol was set as per following cyclic conditions: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 45 s, annealing at 61°C for 30 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The amplified product was digested using 1U of restriction enzyme *Nco*I (Fermentas, USA) by incubating the reaction mixture overnight at 37°C. Restriction products (homozygous AA=97+20, homozygous GG=117 and heterozygous GA=117+97+20) were analyzed on 3% agarose gel and photographed on a gel documentation system. The genotypic results were further validated by Direct Sequencing 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 frequencies 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 $P < .05$.

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[K5]. 79

Table 1. Demographic and clinical parameters of all cases

Parameter	All Cases
Demographic	
Age (years)	47.8±14.9
Gender (Male %)	67
Clinical	
Systolic BP (mmHg)	147±15.3
Diastolic BP (mmHg)	91.8±9.2
Smoking history (%)	37
Urea (mg/dl)	132.3±59.2
BUN (mg/dl)	63.3±29.8
Creatinine (mg/dl)	6.49±3.26
Serum proteins (g/dl)	6.31±0.84
Calcium (mg/dl)	7.13±1.5
Phosphorus (mg/dl)	5.18±2.14
eGFR (ml/min/1.73 m ²)	11.38±6.6

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 [K6] ($P=.17$). 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. Genotype/allelic frequency and association between cases and controls are shown in Table 2. Logistic regression analysis showed a positive association of AA genotype with nephropathy related ESRD. The association was still significant after adjusting for age, gender and clinical confounders ($P=.019$).

Table 2. Genotype and allelic frequencies in study subjects [K7]

Model	Genotype/Allele	Cases	Controls	OR (95% CI)	P value
Codominant	GG	139	185	1	Referent
	GA	69	59	1.5 (1.03-2.34)	.05
	AA	14	6	3.1 (1.1-6.2)	.02
	G	347	429		
	A	97	71	1.6 (1.2-2.3)	.002

Interaction P value adjusted for age and gender=.019

3.3 Association between the GG, GA and AA genotypes and clinical variables in cases

We performed logistic analysis for association between clinical parameters across the GG, GA and AA genotypes and observed a positive association between AA genotype and lower age (GG=48.29±12.43, GA=48.57±18.6, AA=38.86±15.29, $P=.008$). We also observed a significant association for low serum proteins (GG=7.75±0.77, GA=6.40±0.85, AA=6.20±0.82, $P=.03$) and calcium (GG=7.35±1.23, GA=7.01±0.85, AA=6.64±1.02, $P=.01$) 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 [19]. TNF- α is also expressed and released by various renal cells [20].

TNF- α secretion is also linked to the stimulation of other pro-inflammatory cytokines that play a crucial role in modulating renal function [21, 22]. Alterations in TNF- α gene has been associated with several autoimmune and infectious diseases [23, 24]. In the present study we analyzed the association [K8] between TNF- α (-308 G $\leftrightarrow$ A) SNP and nephropathy related ESRD. We observed a positive association between the carriers of AA genotype and the disease ($P=.02$). Though the studies investigating this SNP as a predisposing risk factor for nephropathy/ESRD is conflicting [K9] [14, 15, 25-30], our data however is in conformity with the results of several studies [15, 25, 30]. 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 [K10] [30]. 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 ($P=.008$ [K11]). 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 [31]. Studies show different prognostic implications in renal diseases with respect to age [K12] [32]. In older patients, age-related co-morbidities tend to predict global outcomes like mortality whereas in younger patients specific renal outcomes can be predicted [32]. The association between TNF- α -308 AA genotype also correlated significantly with lower serum protein and calcium levels in our study [K13]. This association can be explained by increased urinary loss of protein ($P=.03$) and calcium ($P=.01$) in such cases. Hypocalcemia has been observed more often in ESRD and is quite uncommon in stages preceding ESRD [19]. The increased proteinuria and hypocalcemia in patients with A/A genotype implicate peritoneal membrane dysfunction leading to rapid progression of renal disease [K14].

5. CONCLUSION

Thus, the study implicates that the variation in the promoter region of TNF- α gene resulting in G to A transition may predispose nephropathy patients to rapid development/progression of ESRD. Screening this genetic variation can be a useful marker for risk assessment and disease management in such patients [K15].

REFERENCES

1. Schoolwerth AC, Engelgau MM, Hostetter TH, Rufo KH, Chianchiano D, McClellan WM, et al. Chronic kidney disease: a public health problem that needs a public health action plan. *Prev Chronic Dis*. 2006;3(2):A57.
2. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, et al. National Kidney Foundation practice guidelines for chronic kidney disease: Evaluation, classification, and stratification. *Ann. Intern. Med*. 2003;139:137–47.
3. Descamps-Latscha B, Herbelin A, Nguyen AT, Roux-Lombard P, Zingraff J, Moynot A, et al. Balance between IL-1 beta, TNF-alpha, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. *J. Immunol*. 1995;154:882–92.
4. Pereira BJ, Shapiro L, King AJ, Falagas ME, Strom JA, Dinarello CA. Plasma levels of IL-1 beta, TNF alpha and their specific inhibitors in undialyzed chronic renal failure, CAPD and hemodialysis patients. *Kidney Int*. 1994;45:890–6.
5. Bantis C, Heering PJ, Luther Y, Aker S, Kuhr N, Grabensee B, et al. Influence of cytokine gene polymorphisms on focal segmental glomerulosclerosis. *Am. J.Nephrol*. 2004;24:427–31.
6. Johnston CI, Risvanis J, Naitoh M, Tikkanen I. Mechanism of progression of renal disease: Current hemodynamic concepts. *J. Hypertens. Suppl*. 1998;16:S3–7.
7. Sankaran D, Asderakis A, Ashraf S, Roberts IS, Short CD, Dyer PA, et al. Cytokine gene polymorphisms predict acute graft rejection following renal transplantation. *Kidney Int*. 1999;56:281–8.

- 168 8. Klahr S. Mechanisms of progression of chronic renal damage. *J. Nephrol.* 1999;12 (2):S53–62..
- 169 9. Wu TH, Tsai CY, Yang WC. Excessive expression of the tumor necrosis factor-alpha gene in the
170 kidneys of patients with membranous glomerulonephritis. *Zhonghua Yi Xue Za Zhi (Taipei).*
171 1998;61:524–30.
- 172 10. Hutchinson IV PV, Sinott PJ. Genetic regulation of cytokine synthesis: Consequences for acute
173 and chronic allograft rejection. *Graft.*1998;1:186–92.
- 174 11. Hampe J, Shaw SH, Saiz R, Leysens N, Lantermann A, Mascheretti S, et al. Linkage of
175 inflammatory bowel disease to human chromosome 6p. *Am. J. Hum. Genet.* 1999;65:1647–55.
- 176 12. Abboud HE. Growth factors in glomerulonephritis. *Kidney Int.* 1993;43:252–67.
- 177 13. Tuglular S, Berthoux P, Berthoux F. Polymorphisms of the tumour necrosis factor alpha gene at
178 position -308 and TNFd microsatellite in primary IgA nephropathy. *Nephrol. Dial. Transplant.*
179 2003;18:724–31.
- 180 14. Buraczynska K, Koziol-Montewka M, Majdan M, Ksiazek A. Polymorphisms of tumor necrosis
181 factor and myeloperoxidase genes in patients with chronic renal failure on peritoneal dialysis.
182 *Mol. Diagn.* 2003;7:175–80.
- 183 15. Manchanda PK, Kumar A, Kaul A, Mittal RD. Correlation between a gene polymorphism of tumor
184 necrosis factor-alpha (G/A) and end-stage renal disease: A pilot study from north India. *Clin.*
185 *Chim. Acta.* 2006;370:152–7.
- 186 16. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease:
187 evaluation, classification, and stratification. *Am J Kidney Dis.* 2002;39:S1-266.
- 188 17. Levey AS, Stevens LA. Estimating GFR using the CKD Epidemiology Collaboration (CKD-EPI)
189 creatinine equation: more accurate GFR estimates, lower CKD prevalence estimates, and better
190 risk predictions. *Am J Kidney Dis.* 2010;55(4):622-627.
- 191 18. Oregon-Romero E, Vazquez-Del Mercado M, Ruiz-Quezada SL, Navarro-Hernandez RE,
192 Rangel-Villalobos H, Martinez-Bonilla G, et al. Tumor necrosis factor alpha-308 and -238
193 polymorphisms in rheumatoid arthritis. Association with messenger RNA expression and sTNF-
194 alpha. *J Investig Med.* 2008;56:937-943.
- 195 19. Navarro-Gonzalez JF, Jarque A, Muros M, Mora C and Garcia J. Tumor necrosis factor-alpha as
196 a therapeutic target for diabetic nephropathy. *Cytokine Growth Factor Rev* 2009;20:165-173.
- 197 20. Schulz S, Schagdarsurengin U, Suss T, Muller-Werdan U, Werdan K and Glaser C. Relation
198 between the tumor necrosis factor-alpha (TNF-alpha) gene and protein expression, and clinical,
199 biochemical, and genetic markers: age, body mass index and uric acid are independent
200 predictors for an elevated TNF-alpha plasma level in a complex risk model. *Eur Cytokine Netw.*
201 2004;15:105-111.
- 202 21. Baud L, Fouqueray B, Philippe C, Amrani A. Tumor necrosis factor alpha and mesangial cells.
203 *Kidney Int.* 1992;41:600–3.
- 204 22. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N. Engl. J. Med.*
205 1994;331:1286–92.
- 206 23. Hajeer AH and Hutchinson IV. Influence of TNF alpha gene polymorphisms on TNFalpha
207 pro-duction and disease. *Hum Immunol* 2001;62:1191-1199.
- 208 24. Hollegaard MV and Bidwell JL. Cytokine gene polymorphism in human disease: on-line
209 databases, Supplement 3. *Genes Immun.* 2006;7:269-276.

- 210 25. Lee SH, Lee TW, Ihm CG, Kim MJ, Woo JT, Chung JH. Genetics of diabetic nephropathy in type
211 2 DM: Candidate gene analysis for the pathogenic role of inflammation. *Nephrology (Carlton)*
212 2005;10:S32–6.
- 213 26. Vázquez-Huerta DI, Alvarez-Rodríguez BA, Topete-Reyes JF, Muñoz-Valle JF, Parra-Michel R,
214 Fuentes-Ramírez F, et al. Tumor necrosis factor alpha -238 G/A and -308 G/A polymorphisms
215 and soluble TNF- α levels in chronic kidney disease: correlation with clinical variables. *Int J Clin*
216 *Exp Med*. 2014;15;7(8):2111-9
- 217 27. Buraczynska M, Mierzicki P, Buraczynska K, Dragan M, Ksiazek A. Tumor necrosis factor-alpha
218 gene polymorphism correlates with cardiovascular disease in patients with end-stage renal
219 disease. *Mol Diagn Ther*. 2007;11(4):257-63.
- 220 28. Singh K, Prasad KN, Mishra P, Singh SK, Kharwar NK, Prasad N, et al. Association of tumour
221 necrosis factor- α polymorphism in patients with end stage renal disease. *Nephrology (Carlton)*.
222 2015 Jun;20(6):387-91. doi: 10.1111/nep.12398.
- 223 29. O'Hare AM, Choi AI, Bertenthal D, Bacchetti P, Garg AX, Kaufman JS, et al. Age affects
224 outcomes in chronic kidney disease. *J Am Soc Nephrol*. 2007;18(10):2758-65.
- 225 30. Goodwin JS: Embracing complexity: A consideration of hypertension in the very old. *J Gerontol A*
226 *Biol Sci Med Sci*. 2003;58:653–658.
- 227 31. Gussekloo J, van Exel E, de Craen AJ, Meinders AE, Frolich M, Westendorp RG: Thyroid status,
228 disability and cognitive function, and survival in old age. *JAMA*. 2004;292:2591–2599.
- 229 32. Langman CB, Cannata-Andía JB. Calcium in chronic kidney disease: myths and realities.
230 Introduction. *Clin J Am Soc Nephrol*. 2010;5(1):S1-2. doi: 10.2215/CJN.06140809.
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