Relationship of Tumor Necrosis Factor-α 308 (G/A) Gene Polymorphism with Risk of Coronary Artery Disease in Hemodialysis Patients

Navid Kalani[*], Zeinab Fattahjahromi, Aghdas Shadmehr

Department of Cardiology, Jahrom University of Medical Sciences, Jahrom, Iran

ABSTRACT

Background: Prevalence and incidence of chronic renal failure is increasing and it has been considered as a threading factor in the worldwide. Cardiovascular diseases has been considered as the cause of more than half of death in hemodialysis patient. It has been shown that systemic inflammation has a key role in this issue. TNFα is a mediator cytokine of inflammation response which has a key role in pathophysiology of vascular disease caused by metabolism of lipids and obesity and insulin resistance. The aim of this study was to assess the association of polymorphism -308G/A gene TNF alpha and severity and vulnerability of cardiovascular disease in hemodialysis patient.

Materials and methods: In this case control study, we had three groups of participants; 41 hemodialysis patient with cardiovascular risk factors and 41 hemodialysis patient without cardiovascular risk factors and also 41 healthy participants without cardiovascular risk factors as control group. DNA extraction was done with the specific Kit for the extraction of DNA. PCR was used for detection of polymorphism -308G/A and then Nocl enzyme was added to each polymorphism for slicing and then electrophoresis was used for confirmation of enzyme slicing. Date were gathered and analyzed with SPSS v 15.

Results: There is no significant difference between the 3 group regarding genotype AA distribution and frequency of Allel A (p value>0.05).

Conclusion: The results of this study demonstrate that there is not any association between this polymorphism and cardiovascular risk in Iranian population. Other studies with larger samples are beneficent in order to detect the role of this polymorphism.

Keywords: Polymorphism-308G/A; TNF alpha; Atherosclerosis; Hemodialysis patient

INTRODUCTION

The prevalence of Chronic Kidney Disease (CKD) is rising worldwide. CKD is currently considered a global health crisis. Although life expectancy of CKD patients has increased in recent years due to advanced dialysis techniques, CKD is a high risk factor for cardiovascular disease and advanced atherosclerosis that contribute to increased mortality rates in CKD patients. Various studies have suggested 10 to 20 times higher mortality rates in patients with Coronary Artery Disease (CAD) undergoing dialysis than normal people although the participants were divided into various categories and were defined by the matching variable (age, gender, race and incidence of diabetes). Recent evidence suggests that systematic inflammation plays a significant role in CKD. Serologic evidence of an activated inflammatory response was estimated in 30% to 50% of patients with End-Stage Renal Disease (ESRD).It is acknowledged that chronic inflammation has a significant role in pathogenesis of atherosclerotic lesions of vascular valve, cardiovascular diseases, mortality and morbidity. Studies have

shown that levels of pre-inflammatory cytokines in patients undergoing dialysis are 8 to 10 times higher than healthy control. High cytokine levels in dialysis patients are due to loss of renal function, uremia and its complications including oxidative stress, fluid overload, susceptibility to infection, dialysis fluid contamination and induced leukocyte activation caused by dialysate impurities and fistula or graft infection. Cytokines are involved in onset of immune and inflammatory responses. TNF-α is one of the most important cytokines mediating inflammatory responses. It plays an important role in pathophysiology of vascular diseases through lipid metabolism, obesity and insulin resistance. It is also acknowledged that high TNF-α levels lead to development of myocardial ischemia and stroke. Progression of atherosclerotic plaques leads to formation of fibrotic caps that largely increases TNF-α enzymatic activity, which triggers platelet adhesion to the Extracellular Matrix (ECM). It is a key step in development of thrombosis.

TNF-α is involved in induction of acute phase reactants. It has a significant relationship with C-Reactive Protein (CRP). CRP is regarded as the prototype of acute-phase proteins in humans that correlates with inflammatory disease. It is also a predictor of cardiovascular mortality in the general population. Acute phase response or systemic response to inflammation and tissue injury is regulated by either enhanced synthesis of positive acute phase proteins (e.g. CRP and fibrinogen) or suppressed secretion of negative acute phase proteins (e.g. albumin and transferrin). Circulating products of these proteins are risk factor for kidney diseases given different mechanisms of these products that contribute to in pathogenesis of the disease. Genetic differences in cytokine products determine interpersonal differences in immune responses. The root of these genetic differences is unknown. However, polymorphism is a reliable and valid genetic factor involved in individual differences. Szebo et al. studied Tumor Necrosis Factor-alpha (TNF-α) polymorphism in atherosclerotic patients and found association of this polymorphism with incidence of myocardial infarction and stroke in atherosclerotic diabetic patients and atherosclerotic non-diabetic patients. The results showed higher incidence of cardiovascular events in patients with mutant TNF-α. Therefore, this genotype is a significant clinical diagnostic and therapeutic marker. Bennett et al. studied the relationship of serum levels of TNF-α and TNF-α promoter polymorphism with the risk of myocardial infarction. The results indicated that high levels of TNF-α increased the risk of myocardial infarction. The risk of myocardial infarction was higher in men with elevated TNF-α levels. Zimmerman et al. also hypothesized that inflammation increased the risk of cardiovascular disease and mortality in hemodialysis patients. They monitored and measured serum lipid profile, apolipoprotein A-I and B, lipoprotein a, fibrinogen and serum albumin levels in comparison with CRP and serum amyloid a (sensitive markers for acute phase response) in two years [1]. The results indicated that a significant number of hemodialysis patients showed an acute phase response, which was closely related to high levels of atherogenic risk factors and cardiovascular death.

Despite these studies, some other studies have reported different results. For example, Banerji et al. studied the relationship between inflammatory gene polymorphism and Coronary Artery Disease (CAD) in the Indian population. They found Single Nucleotide Polymorphism (SNP) present in the genes encoding CD14, TNFα, interleukin-1α, interleukin-VI, PSMA6, and PDE4D and potential role of SNP in vulnerability to CAD [2]. None of the SNPs had a significant relationship with CAD before and after modulating and matching for confounding factors (e.g. age, gender, blood pressure, smoking, and diabetes). Since cardiovascular disease is the leading cause of death for nearly half of all deaths in hemodialysis patients, the present study aimed to determine the relationship of TNF-α polymorphism with vulnerability and severity of cardiovascular disease in hemodialysis patients.

MATERIALS AND METHODS

Statistical population

This was a case-control study. The sample size was determined using statistical methods. The case consisted of 41 hemodialysis patients with a history of coronary artery disease with 50% stenosis in at least one coronary vessel diagnosed by angiography and 41 hemodialysis patients without coronary artery disease who visited the dialysis department [3]. Forty-one healthy individuals were also selected as control. All of them underwent necessary tests and full examinations and showed normal test results. They were matched in terms of such confounding factors as diabetes and polycystic kidney. The individuals in case and control were matched for age, gender and race. Patients with history of liver disease, malignancy, clinical signs of coagulation disorder and acute intoxication (e.g. amphetamine) were excluded from the study. A questionnaire consisting of demographic data, history of disease and intake of medication in the last months and health status of patients' family was used for data collection. Blood samples were collected in EDTA-containing tubes [4]. Written consent forms were collected from all the participants (a description of the study, advantages of the study, sampling method or other services, confidentiality of data, and the right to quit the study). The study was approved by the Ethics Committee.

Genotype determination

DNA of collected blood samples was extracted using DNPTM DNA extraction kit (Neda Fan Corporation). DNA extract quantity (concentration and extraction efficiency) and quality were assessed by nanodrop machine. Genotype of Rs3856806PCR-RFLP polymorphism was determined using PCR-RFLP method [5]. For this purpose, the fragments containing the above polymorphism were first amplified by the specific primers and the conditions presented in Table 1.

Table 1: Specific primers and conditions.

Sequence of specific primers of Rs 3856806 polymorphism and polymerase chain reaction conditions	
Polymorphism (Rs No.)	Primer sequence
TNF-α G308A	AGGCAATAGGTTGAGGGCCAT'
(Rs 3856806)	5'_TCCTCCCTGCTCCGATTCCG

Relevant articles were used to select forward and reverse primers. To ensure the sequence of these primers, DNA sequences of encoding genes of Rs 3856806 polymorphism were collected from the Gene Bank database. Both primers were examined using available information and gen runner. DNA sequences were checked in the blast. PCR was performed to amply the fragments. For this purpose, 150 ng of genomic DNA, 2 mM $MgCl_2$, 1.5 μM dNTPs, 1 pM of each primer and 2 units of Taq DNA polymerase were mixed [6]. Sterile distilled water was added to the mixture to increase volume of the solution to 25 micro liters. Eppendorf machine was used for PCR with the protocol of initial denaturing at 94°C for five minutes, secondary denaturing at 94°C for 35 seconds, annealing at 59°C for 40 seconds and extending at 72°C for 40 seconds, and 35 repeated cycles. Final extending temperature was 72°C in 5 minutes. PCR product was digested with NcoI restriction enzyme at 37°C and incubated for 16 hours [7]. The digested fragments were separated by gel electrophoresis. The mutant homozygote had AA genotype and mutant heterozygote had AG genotype. The wild form had GG genotype. A band of 107 bp was found in case that the restriction enzyme did not cut the DNA fragment (A allele) and a band of 20 bp was found in case that the restriction enzyme cut the DNA fragment (87-A allele).

Statistical analysis

Quantitative variables were presented by mean and standard deviations. Qualitative variables were presented by percent. All statistical methods were performed using SPSS v. 15. t-test was used to compare quantitative variables between control and case. *Chi-square* was used to compare qualitative variables. ANOVA was used to compare quantitative variables in different genotypes. P-values less than 0.05 were considered statistically significant.

RESULTS

As mentioned in the above section, the case consisted of 41 hemodialysis patients with CAD and 41 hemodialysis patients without CAD. Control also consisted of 41 healthy participants. ANOVA results showed no significant difference in average age of the participants. P-value in hemodialysis patients without CAD compared to hemodialysis patients with CAD was 0.212. P-value in hemodialysis patients without CAD compared to control was 0.179. P-value in hemodialysis patients with CAD compared to control was 0.923. Frequencies of distribution of genotypes in Rs 3856806 polymorphism in hemodialysis patients with CAD and hemodialysis patients without CAD (Table 2) were AA genotype 9 (22.5%), 11 (27.5%) (P=0.606), AG genotype 16 (40.0%), 13 (5.2%) (P=0.486) and GG genotype 15 (37.5%), 16 (40%) (P=reference). There was no significant difference in the two groups. The frequencies of allele A in the two groups were 34 (42.5%) and 35 (43.8%) respectively. No significant difference was found between the two groups (P=0.873). Frequencies of distribution of genotypes in Rs 3856806 polymorphism in hemodialysis patients with CAD compared to control (Table 3) were AA genotype 9 (22.5%), 6 (15%) (P=0.353), AG genotype 16 (40.0%), 16 (40%) (P=1.000) and GG genotype 15 (37.5%), 18 (45%) (P=Reference). No significant difference was found between the two groups. Frequencies of allele A in these two groups were 34 (42.5%) and 28 (35%) respectively. The difference was not significant (P=0.331). Frequencies of distribution of genotypes in Rs 3856806 polymorphism in hemodialysis patients without CAD compared to control (Table 4) were AA genotype 11 (27.5%), 6 (15%) (P=0.177), AG genotype 13 (32.5%), 16 (40%) (p=0.486) and GG genotype 16, (40%) 18 (45%) (P=Reference). There was no significant difference between the two groups. The frequencies of allele A in these two groups were 35 (43.8%) and 28 (35%). The difference was not significant (P=0.331).

Table 2: Distribution of G/A308 genotype polymorphism in hemodialysis patients with and without CAD.

Genotype	Hemodialysis patients with the risk of CAD (n=40)	Hemodialysis patients without the risk of CAD (n=40)	OR	Pvalue
AA	9 (22.5%)	11 (27.5%)	0.765 (0.277-2.114)	0.606
AG	16 (40.0%)	13 (5.2%)	1.385 (0.544-3.458)	0.486
GG	15 (37.5%)	16 (40%)	Reference	Reference

AG+AA	25 (62.5%)	24 (60%)	11.1 (0.452-2.733)	0.818
Frequency of A allele	34 (42.5%)	35 (43.8%)	0.950 (0.508-1.777)	0.873

Table 3: Comparison of results in hemodialysis patients with CAD compared to control.

Genotype	Hemodialysis patients with the risk of CAD (n=40)	Control (n=40)	OR	P-value
AA	9 (22.5%)	6 (15%)	1.80 (0.521-6.218)	0.353
AG	16 (40.0%)	16 (40%)	1.00 (0.409-2.45)	1
GG	15 (37.5%)	18 (45%)	Reference	Reference
AG+AA	25 (62.5%)	22 (45%)	1.364 (0.558-3.33)	0.496
GG	15 (37.5%)	0 (45%)	Reference	Reference
Frequency of allele A	34 (42.5%)	28 (35%)	1.373 (0.725-2.599)	0.331

Table 4: Comparison of results in hemodialysis patients without CAD compared to control.

Genotype	Hemodialysis patients without the risk of CAD (n=40)	Control (n=40)	OR	P-value
AA	11 (27.5%)	6 (15%)	2.149 (0.7076.53)	0.177
AG	13 (32.5%)	16 (40%)	0.722 (0.2891.804)	0.486
GG	16 (40%)	18 (45%)	Reference	Reference
AG+AA	24 (60%)	22 (55%)	1.227 (0.5050.982)	0.651
GG	16 (40%)	18 (45%)	Reference	Reference
Frequency of allele A	35 (43.8%)	28 (35%)	1.373 (0.7252.599)	0.331

DISCUSSION

The participants consisted of 41 hemodialysis patients with CAD (diagnosed by a cardiologist and angiography) and 41 hemodialysis patients without CAD who visited the dialysis department of health centers in Jahrom Town and 41 healthy people [8]. The samples with clear bands were confirmed in qualitative assessment of extracted DNA using 1% agarose gel. Agarose gel electrophoresis (2%) of PCR products was performed to ensure amplification of desired fragments of TNF-α gene. Clear bands appeared at the site of predefined base pairs. These fragments were selected for the study. Then, PCR products were digested by the given enzyme. Agarose gel electrophoresis (2%) was performed to detect polymorphism in the fragments. Statistical test results indicate no significant difference between hemodialysis patients with CAD and hemodialysis patients without CAD (P=0.606) in terms of

distribution of mutant AA homozygote. No significant difference was also found between the two groups in terms of mutant AG heterozygote (P=0.486). Frequencies of allele A in G/A308 polymorphism in hemodialysis patients with the risk of CAD and hemodialysis patients without CAD were 42.4% and 43.8% respectively. The difference between the two groups was not significant (OR=0.950; 95% CI=0.508-1.777; P=0.873) [9]. There was no significant difference in distribution of mutant AA homozygote between hemodialysis patients with CAD and control (P=0.353). Frequency of mutant AG heterozygote was identical in both groups (P=1.000). Frequencies of allele A in G/A308 polymorphism in hemodialysis patients with CAD and control were 42.5% and 35%. Despite higher frequency of allele A in hemodialysis patients with CAD, no significant difference was found between the two groups (OR=1.373; 95% CI =0.725-2.599; P=0.331). Distribution of mutant AA homozygote

between hemodialysis patients without CAD and control did not show a significant difference (P=0.177).

The frequency of mutant AG heterozygote was higher in control compared to hemodialysis patients without the risk of CAD but the difference was not significant (P=0.486). Frequencies of allele A in G/A308 polymorphism in hemodialysis patients without CAD and control were 43.8% and 35% respectively. Although the frequency of allele A was higher in hemodialysis patients without CAD compared to control, the difference was not significant (OR=1.373; 95% CI=0.725-2799; P=0.331). Logistic regression analysis on distribution of G/A308 polymorphism by gander showed a significant different in AG heterozygote between hemodialysis patients with CAD and control (P<0.05). The gender factor was controlled in logistic regression to run analysis on age. Regression analysis on distribution of AG genotype in 45-59.9 and 30-44.9 age groups showed no significant difference between hemodialysis patients with CAD compared with hemodialysis patients without CAD (P<0.05).

There was no significant relationship between other groups and age groups. Analysis of frequency of genotypes and allele A in G/A308 TNF-α promotor polymorphism revealed different results in different populations. Vaidyanathapuran studied AA and AG genotypes in hemodialysis patients and found out significantly higher comorbidity and lower functional score (based on Karnofsky index) in hemodialysis patients with higher TNF-α levels compared to lower TNF-α level. Therefore, TNFα promoter polymorphism in ESRD patients had a strong association with comorbidity and performance, biological and nutritional indices [10]. Buraczynska studied the association of this polymorphism with chronic renal failure in peritoneal dialysis. There was no association between patients and healthy individuals in terms of this polymorphism.

However, the frequency of this polymorphism in healthy women was significantly higher than women with acute renal failure. Bhanushali et al. found a significant difference in sequence of 308G/A TNF-α between CAD patients and control in the Indian population. However, no association was found between this polymorphism and increased risk of CAD in final genotype analysis. Sobti et al. found no strong association between CAD and this polymorphism in the Indian population (P<0.0001; OR 0.194, 95% CI 0.103-0.365). An association was found between metabolic syndrome and DM2. AG genotype (P=0.002; OR 4.484) and AG+AA (P=0.002; OR 4.255) had a strong association with metabolic syndrome and DM2. AA and AG genotypes (P=0.001; OR 5.497) had a strong correlation with obesity in men but AG genotype (P=0.001) had a strong association with obesity in women. AA genotype (P=0.043 OR 3.352) and AG genotype (P=0.001 OR5.011) had a strong relationship with metabolic syndrome and obesity. AG genotype protected against CAD in obese patients (P<0.0001) [11-13]. TNF-α 308G/A heterozygote genotype is probably an important risk factor for metabolic syndrome in comorbidity with obesity and DM2 but it probably protects against CAD in comorbidity with obesity and DM2. Sobti et al. showed higher frequency of TNF-α 308 A allele in CAD patients compared to healthy individuals in Italy (P=0.045). Following the classification of risk

factors for CAD, analysis results showed that CAD patients with DM (P=0.042) and CAD patients without hypertension (p=0.0495) had a higher frequency of sequence of TNFα-308AA genotype than normal people. Evidence suggested TNFα-308G/A genotype contribute to progression of CAD disease. Lower frequency of allele A suggested progression of CAD in patients with comorbidities. However, Koch showed that this polymorphism was not involved in restenosis, morbidity or MI. Kumar et al. compared 5651 patients with 5792 healthy individuals and found the significant protective role of this polymorphism in CAD (P=0.03). Analysis results showed that TNF-α 238G/A polymorphism had a stronger relationship with the risk of ischemic stroke in the Caucasians compared to the Asians [14,15]. TNF-α 308G/A polymorphism seems to have a more protective role in ischemic stroke in the Asian population compared to the Caucasian population.

CONCLUSION

The results of this study are consistent with previous findings on the role of 308 G/A TNF-α gene polymorphism in susceptibility to heart disease. They suggest that this polymorphism is not associated with heart disease in hemodialysis patients in Jahrom Town. The results of this study suggest that this polymorphism has no association with the risk of heart disease in the Iranian population and is not involved in progression of this disease. It is recommended that this polymorphism be studied in larger sample size and population to determine definitive role of this polymorphism in CAD.

REFERENCES

1. Hosseinpanah F, Kasraei F, Nassiri AA, Azizi F. High prevalence of chronic kidney disease in Iran: A large population-based study. BMC Public Health. 2009;9(1):1-8.

2. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol: JASN. 1998;9(12):16-23.

3. Pupim LB, Himmelfarb J, McMonagle E, Shyr Y, Ikizler TA. Influence of initiation of maintenance hemodialysis on biomarkers of inflammation and oxidative stress. Kidney Int. 2004;65(6): 2371-2379.

4. Jacobs P, Glorieux G, Vanholder R. Interleukin/cytokine profiles in haemodialysis and in continuous peritoneal dialysis. Nephrol Dial Transplan. 2004;19(5):41-45.

5. Kaizu Y, Kimura M, Yoneyama T, Miyaji K, Hibi I, Kumagai H. Interleukin-6 may mediate malnutrition in chronic hemodialysis patients. Am J Kidney Dis. 1998;31(1):93-100.

6. Szabo GV, Acsady G. Tumornecrosis-factor-α 308 GA polymorphism in atherosclerotic patients. Pathol] Oncol Res. 2011;17:853-857.

7. Settin A, Abdel-Hady H, El-Baz R, Saber I. Gene polymorphisms of TNF-α-308, IL-10-1082, IL-6-174, and IL-1Ra VNTR related to susceptibility and severity of rheumatic heart disease. Pediatr Cardiol. 2007;28:363-371.

8. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int. 1999;55(2):648-658.

9. Bennet AM, Maarle MC, Hallqvist J, Morgenstern R, Frostegard J, Viman B, et al. Association of TNF-α serum levels and TNFA promoter polymorphisms with risk of myocardial infarction. Atherosclerosis. 2006;187(2):408-414.

10. Banerjee I, Pandey U, Hasan OM, Parihar R, Tripathi V, Ganesh S. Association between inflammatory gene polymorphisms and coronary artery disease in an Indian population. J Thrombo Thrombol. 2009;27:88-94.

11. Balakrishnan VS, Guo D, Rao M, Jaber BL, Tighiouart H. Cytokine gene polymorphisms in hemodialysis patients: Association with comorbidity, functionality, and serum albumin. Kidney international. 2004;65(4):1449-1460.

12. Buraczynska K, Koziol-Montewka M, Majdan M, Ksiazek A. Polymorphisms of tumor necrosis factor and myeloperoxidase genes in patients with chronic renal failure on peritoneal dialysis. Mol Diagnos. 2003;7:175-1780.

13. Bhanushali AA, Das BR. Promoter variants in interleukin-6 and tumor necrosis factor alpha and risk of coronary artery disease in a population from Western India. Indian J Hum Genet. 2013;19(4): 430.

14. Sobti RC, Kler R, Sharma YP, Talwar KK, Singh N. Risk of obesity and type 2 diabetes with tumor necrosis factor-α 308G/A gene polymorphism in metabolic syndrome and coronary artery disease subjects. Mol Cell Biochem. 2012;360:1-7.

15. Koch W, Kastrati A, Bottiger C, Mehilli J, von Beckerath N, Schomig A. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis. 2001;159(1):137-144.