Association of tumor necrosis factor-α polymorphisms with susceptibility and clinical outcomes of rheumatic heart disease

Amal A. Mohamed, MSc, MD, *Laila A. Rashed,* MSc, MD, *Saher M. Shaker,* MSc, MD, *Rasha I. Ammar,* MSc, MD.

ABSTRACT

الأهداف: دراسة علاقة التعدادات الشكلية لجين معامل نخر الأورام ألفا (TNF-α) بمرض روماتيزم القلب (RHD) وتلف الصمامات، وتأثير هذا الجين على مستوى معامل نخر الأورام في الدم ونتيجة المرض.

الطريقة: أجريت هذه الدراسة المقطعية العرضية في الفترة مابين ديسمبر 2008م حتى أكتوبر2009م مستشفى العيني – جامعة القاهرة – مصر، على 80 طفل مصري مصاب بمرض روماتيزم القلب بعد إقصاء المصابين بأمراض أخرى أو أية مضاعفات و50 طفل طبيعي كمجموعة ضابطة. تم الحصول علي عينات من الدم (5مل) للبحث عن التعدادات الشكلية للجين بواسطة تفاعل البلمرة المتسلسل، وقياس معامل نخر الأورام في مصل الدم بطريقة الإليزا.

النتائج: أن مستوي معامل نخر الأورام في الدم مرتفع ارتفاع ذو دلالة إحصائية في مجموعة المرضى المصابين بمرض روماتيزم القلب RHD عن المجموعة الضابطة p=0.00003. كانت الأنواع الجينية للأدينين p=0.036 238AA- وp=0.003 308AA- مرتفعة في مجموعة المرضى عن المجموعة الضابطة وكانت مصاحبة بزيادة في نسبة حدوث المرض. تشترك الأشكال الجينية في رفع حساسية مرض روماتيزم القلب للأنواع الجينية 238AA- و الأدينين جوانين (AG) معدل إحتمالي= 4.72، 95%CI 2.03-11.05، ومعدل إحصائي p=0.0001. بلغ المعدل الإحتمالي= 2.33 للنمط الجيني 308AA- و AG، والمعدل الإحصائي p=0.035، CI 11.05-5.19. كان النمط الجيني 308AA- مصاحباً لتلف الصمام المترالي p=0.001، وتلف الصمامات المتعددة p=0.003، والحالات المتوسطة p=0.001، والشديدة p<0.001. كان النمط الجيني 238AA- مصاحباً لتلف الصمام المترالي p=0.04، والحالات الشديدة مقارنة بمجموعة التحكم p=0.05.

خاتمة: نستنتج مما سبق أن التعدادات الشكلية TNF-α-238G/A و 308G/A- لجين معامل تحلل الأورام وجد إنها مصاحبة لحدوث مرض روماتيزم القلب RHD وزيادة معامل تحلل الأورام في الدم ووجد أن لها علاقة بتلف الصمامات وشدة المرض.

Objectives: To examine the association of tumor necrosis factor-alpha (TNF-α) gene polymorphisms with rheumatic heart disease (RHD) and valve damage, and their influence on TNF-α production and disease outcome.

Method: We performed this cross-sectional study at Kasr El-Aini Hospital, Cairo University, Cairo, Egypt, from December 2008 to October 2009. Eighty children with chronic RHD and valve affection, and 50 controls were included. Patients with any other diseases or complications were excluded. Blood samples (5 ml) were collected. Genotyping for TNF-α polymorphisms was performed by the polymerase chain reaction-restriction fragment length polymorphism method. Serum TNF-α was measured by enzyme-linked immunosorbent assay.

Results: Serum TNF-α was significantly increased in RHD compared with controls (p=0.00003). The TNF-α -238 adenine (AA) (p=0.036) and -308AA (p=0.003) genotypes were more frequent in RHD patients than in controls, and were associated with increased production of TNF-α (p=0.00001 for 238AA) and (p=0.001 for 308AA). Both polymorphisms contributed to increased susceptibility for RHD (-308AA and adenine guanine (AG), odds ratio [OR]=4.72 [95% confidence interval [CI] 2.03-11.05], p=0.0001); (-238 AA and AG, OR=2.33 [CI: 1.05-5.19], p=0.035). The presence of -308AA was associated with mitral (p=0.001) and multivalvular (p=0.003) lesions and was more prevalent in moderate (p=0.001), and severe (p<0.001) cases than in controls. The -238AA variant was associated with mitral lesions (p=0.04) and severe cases (p=0.05) as compared with controls.

Conclusion: The TNF-α-238G/A and -308G/A polymorphisms were associated with susceptibility to RHD and increased production of TNF-α. Both polymorphisms were related to valve damage, and a more severe outcome of RHD.

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From the Departments of Clinical and Chemical Pathology (Mohamed), Biochemistry (Rashed), and Pediatrics (Shaker, Ammar) Faculty of Medicine, Cairo University, Cairo, Egypt.

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Address correspondence and reprint request to: Dr. Amal A. Mohamed, Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University 11562, Cairo, Egypt. Tel. +2 (10) 2412882. Fax. +2 (23) 5837176. E-mail: amal_abd_elwahab @yahoo.com

Rheumatic fever (RF) is an inflammatory disease caused by the group A streptococcus pyogenes that follows a non-treated throat infection in susceptible children.[1,2] The major importance of RF is its ability to cause progressive and permanent lesions of the heart valves leading to rheumatic heart disease (RHD).[3] Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine produced by monocytes, macrophages, and thymus (T) and bursa (B) lymphocytes.[4] It was reported that blood mononuclear cell cultures from rheumatic children produced more TNF-α than those from controls.[5] Moreover, previous work[6] has shown, through the immunohistochemical analysis of surgical heart tissue fragments from patients with RHD, a predominance of cells positive for TNF-α in lesion sites in both myocardium and valves. Two gene polymorphisms at positions -308 guanine (G)/adenine (A) and -238G/A have been described within the promoter region of the TNF-α gene on chromosome 6, which are associated with susceptibility to a range of autoimmune disorders.[7] Several studies[1,3] on the association of TNF-α-308G/A and -238G/A gene polymorphisms with risks of RHD have been carried out, however, the results have been controversial, and there has been a discrepancy among the different studied populations.[4] In the present study, we aim to examine the possible association of -238G/A polymorphism of the TNF-α gene with susceptibility to RHD, valve damage, and disease outcome, and the influence of -238G/A and -308G/A on serum TNF-α levels in Egyptian children with RHD. We also examined -308G/A polymorphism in a larger sample of Egyptian children.

Methods. This cross-sectional study was performed from December 2008 to October 2009 in the Department of Clinical and Chemical Pathology, Kasr El-Aini Hospital, Cairo University, Cairo, which is the main referral hospital in Egypt. Ethical approval was obtained from the Research Ethics Committee of the Clinical and Chemical Pathology Department, and parental informed consent was obtained. The study included 80 pediatric patients with RHD (mean age ±standard deviation [SD]=11.8 years [±4.2], and a boy: girl ratio of 52:28). Diagnosis of RHD and evaluation of valvular damage using echocardiography and angiography were based on Jones criteria and consensus guidelines on pediatric acute RF and RHD.[8] All patients had chronic RHD, and one or more valve affection. Patients with complications such as, congestive heart failure, or atrial fibrillation, and those with any other diseases, or inflammatory conditions in addition to RHD were excluded. Patients were classified as having either mitral valve damage (MVD) (51 cases [64%]), or multivalvular lesions (MVL) (29 cases [36%]). Severity of valve lesions was graded according to echocardiographic findings as mild (17 patients [21%]), moderate (39 patients [49%]), or severe (24 patients [30%]). Fifty healthy unrelated children with matched age and gender were enrolled as a control group. They were selected from those attending the dental outpatient clinic for routine check up. All of them had negative family history of RHD. For each patient and control, venous blood samples (5 ml) were collected in plain tubes (2 ml), and ethylene diamine tetraacetic acid (EDTA) tubes (3 ml). Genomic DNA was obtained using the salting-out technique.[9] The DNA and serum samples were stored at -20°C until analyzed. Analysis of serum TNF-α was carried out by solid phase sandwich enzyme immuno-assay (ELISA). The kit was supplied by Quantikine (R & D Systems, Minneapolis, MN, USA). The TNF-α-238G/A and -308G/A genotypes were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The TNF-α-238G/A polymorphisms were determined using the forward primer 5'-AAACAGACCACAGACCTGGTC-3,' and the reverse primer 5'-CTCACACTCCCCATCCTCCCGGATC-3' that includes a restriction site for the *Bacillus amyloliquefaciens* HI (*Bam*HI) enzyme. The PCR products were visualized on 3% agarose gel stained with ethidium bromide.[3] The TNF-308G/A polymorphism was analyzed using the forward primer 5'-GAGCAATAGGTTTTGAGCGCCAT-3,' and the reverse primer 5'-GGGACACACAAGCATCAAG-3' to create a restriction site for the *Nco*I enzyme. The PCR products were visualized in 2% agarose gel stained with ethidium bromide.[10]

The Statistical Package for Social Sciences (SPSS Inc, Chicago, IL, USA) version 10.0 for Windows was used for data management and analysis. Genotype and allele frequencies were calculated by direct gene counting, and data were expressed as frequency and percentage. Association between qualitative data was carried out using chi-squared test and odds ratio (OR). Quantitative data were presented as means ± standard deviation (SD) and compared using student t test. Medians (interquartile range) were also presented. Differences with $p<0.05$ were considered significant.

Results. The mean serum TNF-α was significantly higher in the RHD group (mean ± SD: 8.6 pg/ml ± 4.1) compared with controls (mean ± SD: 5.6 pg/ml ± 3.3) ($p=0.00003$). The median (interquartile range) values were 8.1 (5.9-10.1) for RHD, and 5.2 (2.7-8.0) for controls. In the control group, mean serum TNF-α was significantly increased in children with -308AA genotype (mean ± SD: 8.35 pg/ml ± 2.41) compared with carriers of -308G allele (mean ± SD: 5.4 pg/ml ±

3.31) (p=0.001). Similar trends were found in children homozygous for -238A allele (mean ± SD: 9.1 pg/ml ± 2.9) compared with carriers of the -238G allele (mean ± SD: 5.3 pg/ml ± 3.1) (p=0.00001) (**Figure 1**). The median (interquartile range) values were: 8.0 (6.8-9.5) for -308AA genotype; 4.7 (2.7-7.8) for -308G carriers; 9.8 (7.5-11.1) for -238AA genotype; and 4.9 (2.7-7.3) for -238G carriers. The TNF-308 polymorphism analysis showed significantly higher frequencies of A allele (p=0.000005) and AA genotype (p=0.003) in the patient group compared with healthy controls. The presence of G Allele (p=0.001) and guanine guanine (GG) variant (p=0.0001) was more common among controls than the patients group. In the case of -238 polymorphism, patients showed increased frequencies of A allele (p=0.007) and AA genotype (p=0.036) when compared with controls. However, decreased frequencies of G allele (p=0.01), and GG genotype (p=0.035) in patients were found (**Table 1**). Both polymorphisms contributed to increased susceptibility for RHD, the OR for -308AA and AG genotype was found to be 4.72, 95% confidence interval (CI)=2.03-11.05 (p=0.0001), and the OR for -238AA and AG genotype was 2.33, 95% CI=1.05-5.19 (p=0.035). The patients were stratified according to valve lesion into patients with MVD, and patients with MVL. Allele and genotype frequencies of TNF -238 and -308 polymorphisms of these subgroups were compared between them and with the control group. Patients with MVD showed increased frequencies of -308AA (p=0.001) and -238AA (p=0.04), and decreased frequencies of -308GG (p=0.0004) variants when compared with controls. Patients with MVL presented with increased frequencies of -308AA (p=0.003) and decreased frequencies of -308GG (p=0.012), and -238GG (p=0.04) genotypes as compared with controls. No statistical difference was found in the distribution of -238 and -308 polymorphisms between patients with MVD, and those with MVL. Further subdivision of patients with RHD into those with mild, moderate, and severe lesions showed that severe cases presented with significantly higher frequencies of -308AA (p=0.00004) and -238AA (p=0.05) variants as compared with controls, moderate cases presented with higher frequencies of -308AA (p=0.001) than controls, whereas frequencies of -308AA and -238AA were comparable between mild cases (p=0.63) and controls (p=0.06) (**Tables 2 and 3**).

Discussion. The TNF-α is apparently one of the cytokines with an active and prominent role in the pathogenesis of the rheumatic process.[11] The assumption that polymorphisms in the TNF-α gene may influence the clinical outcomes of RHD was based on previous observations that heart infiltrating mononuclear cells from RHD patients positive for TNF-α were more prevalent in both myocardium and valve lesions.[6]

In the current study, we found that both -308AA and -238AA variants of TNF-α gene were more prevalent in RHD patients in comparison with controls, and were associated with increased susceptibility to RHD. In aggrement with our results, the association of the -308A allele with RHD was found in patients from Mexico,[12] Turkey,[11] and Brazil.[3] However, Berdeli et al[13] found no association between -308 polymorphism and

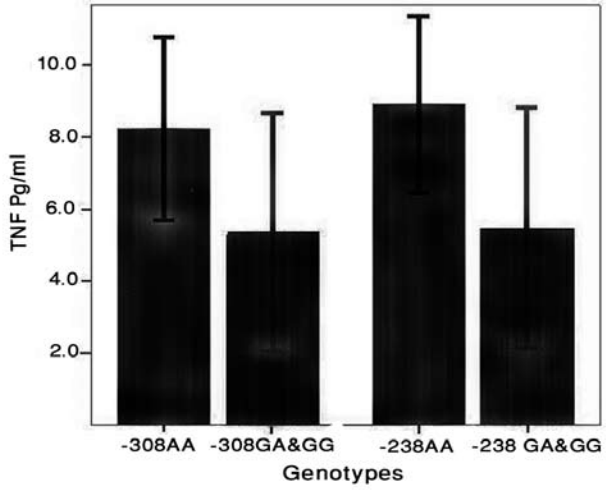

Figure 1 - Mean±standard deviation tumor necrosis factor-alpha (TNF-α) serum levels according to genotypes of the -238 G/A and -308 G/A TNF-α polymorphisms. The -238 AA and -308 AA variants were associated with higher levels of TNF-α compared to -238 GA and GG and -308 GA and GG genotypes. G - guinine, A -adenine

Table 1 - Genotype and allele frequencies of TNF-α -308 G/A and -238 G/A in RHD patients and controls.

Variables	RHD (n=80)	Controls (n=50)	*P*-value
	n (%)		
TNF-α -308 Genotypes			
AA	31 (38.8)*	4 (8.0)	0.003
AG	32 (40.0)	18 (36.0)	0.786
GG	17 (21.2)	28 (56.0)	0.0001
Alleles			
A	94 (58.8)*	26 (26.0)	0.000005
G	66 (41.2)	74 (74.0)	0.0001
TNF-α -238 Genotypes			
AA	17 (21.2)*	3 (6.0)	0.036
AG	39 (48.7)	22 (44.0)	0.728
GG	24 (30.0)	25 (50.0)	0.035
Alleles			
A	73 (45.6)*	28 (28.0)	0.007
G	87 (54.3)	72 (72.0)	0.01

TNF-α - tumor necrosis factor-alpha, RHD - rheumatic heart disease, A - Adenine, G - Guanine, *significantly increased as compared to controls

Table 2 - Tumor necrosis factor -308G/A genotypes in different clinical phenotypes of rheumatic heart disease compared to controls.

Variables	Genotype n (%)			Odds ratio (95% CI) AA, AG versus GG
	AA	AG	GG	
Controls, (n=50)	4 (8.0)	18 (36.0)	28 (56.0)	
Valve lesion				
MVD, (n=51)	20 (39.2)*	21 (41.2)	10 (19.6)†	5.22 (1.98 - 14.07)
MVL, (n=29)	11 (37.9)††	11 (37.9)	7 (24.2)§	4.0 (1.31 - 12.63)
Severity				
Severe, (n=24)	13 (54.2)†	6 (25.0)	5 (20.8)§	4.84 (1.40 - 17.71)
Moderate, (n=39)	16 (41.0)*	17 (43.6)	6 (15.4)*	7.0 (2.26 - 22.65)
Mild, (n=17)	2 (11.8)	9 (52.9)	6 (35.3)	2.33 (0.66 - 8.54)

CI - confidence interval, MVD- mitral valve disease, MVL - multivalvular lesions, A- Adenine, G- Guanine
*p=0.001, †p=0.0004 ††p=0.003 , §p=0.01

Table 3 - Tumor necrosis factor -238G/A genotypes in different clinical phenotypes of rheumatic heart disease compared to controls.

Variables	Genotype n (%)			Odds ratio (95% CI) AA, AG versus GG
	AA	AG	GG	
Controls, (n=50)	3 (6.0)	22 (44.0)	25 (50.0)	
Valve lesion				
MVD, (n=51)	11 (21.6)*	23 (45.1)	17 (33.3)	2.0 (0.83 - 4.85)
MVL, (n=29)	6 (20.6)	16 (55.2)	7 (24.2)*	3.14 (1.03 - 9.88)
Severity				
Severe, (n=24)	6 (25.0)†	10 (41.7)	8 (33.3)	2.0 (0.65 - 6.25)
Moderate, (n=39)	7 (17.9)	20 (51.3)	12 (30.7)†	2.25 (0.86 - 5.96)
Mild, (n=17)	4 (23.5)	9 (52.9)	4 (23.5)	3.25 (0.82 - 13.83)

CI - confidence interval, MVD- mitral valve disease, MVL - multivalvular lesions,
A- Adenine, G- Guanine. *p=0.04, †p=0.05

RF in the Turkish population. Concerning the -238 polymorphism, our data are consistent with the findings of Ramasawmy et al.[3] However, our results differed from that of Hernandez-Pacheco et al[12] where the -238A allele was found to confer protection. Variants of TNF-α may be one of the predisposing risk factors for RHD, which act with other factors, both genetic and environmental in the development of the disease.[3] This might explains the discrepancy of results from association studies carried on populations with different ethnic origins, genetic backgrounds, and variable environmental factors.[14] Other possible causes of discrepancy might be different ages and sample sizes of the studied populations. The frequencies of -308A (26%) and -238A (28%) alleles in the control group of our study were much higher compared withsubjects from Mexico[12] (1.5% for -308A, and 7.6% for -238A), and Brazil[3] (9% for -308A, and 4% for -238A). These conflicting results might be attributed to different ethnic origins of the studied populations. Investigations comparing populations with different ethnic backgrounds have shown significant variations in the allelic frequency.[4] As for Arab populations, our data were different from the prevalence of -308A (8.1%) and -238A (9.7%) alleles in women from Bahrain,[15] and was to some extent comparable with data reported for women from Tunisia[16] (18.5% for -308A, and 21% for -238A alleles). Our control group was small compared withother studies, therefore, further larger studies will be important to accurately represent allelic distribution in the Egyptian population.

In comparing valvular lesion subgroups between each other and with controls, we found that patients with MVD and those with MVL had similar distribution of -238 and -308 polymorphisms, indicating that TNF-α gene shows an overall susceptibility to RHD. However, -308 polymorphisms showed statistically significant associations with mitral and MVL when compared with controls. A similar association was reported in previous

studies.[1,3,12] In the case of -238 polymorphisms, a significant association was found with MVD. Our data are in agreement with Hernandez-Pacheco et al,[12] however, Ramasawmy et al[3] found no association between -238 polymorphisms and MVL. One possible explanation of this discrepancy could be the different ages, sample sizes, and environmental factors of the studied populations. In genetically susceptible individuals, environmental factors, and group A streptococcal infection contribute to the development of RF, as well as differentiation of RF into different clinical phenotypes overtime.[3]

In our study, aortic valve lesions (AVLs) were included in the multivalvular group. Further studies on a larger sample size including AVL as a separate entity is warranted. Our data showed that TNF-α -308AA and -238AA genotypes were significantly associated with susceptibility to a more severe form of RHD disease. The relation between -308 polymorphisms and severity of RHD was suggested by Settin et al[1] and Ramasawmy et al.[3] The progression from benign autoimmunity to autoimmune disease may depend on the balance of inflammatory cytokine production. Increased inflammatory cytokine synthesis can favor autoimmune disease development.[11] In the current study, RHD cases presented with higher serum TNF-α level as compared withcontrols. The pathogenic role of TNF-α in the development of RHD was also reported by Guilherme et al[6] and Gorbunov et al.[17] Our results showed that the -308AA genotype was associated with increased production of TNF-α. Similar findings were reported by Sallakci et al,[11] Lu et al,[18] and Yoon et al.[19] However, a previous study suggested that the TNF-α -308G allele associates with high serum TNF-α levels.[20] On the other hand, Escobar-Morreale et al[21] and Bennet et al[22] found that the production of TNF-α is not influenced by the TNF-α -308G/A polymorphism.

Some studies suggested that the variants of TNF-α alone are not sufficient for disease development, and they are in linkage disequilibrium with other immunologically relevant human leukocyte antigen (HLA) genes.[3] Of interest, many studies have reported a correlation between HLA genotype and TNF-α expression levels.[19,23] Concerning the TNF -238G/A polymorphism, we found that the -238AA variant was associated with higher TNF-α levels as compared withcarriers of the G allele. Similar results were reported in previous studies.[24,25] Bayley et al[24] suggested that the region between -254 and -230 of chromosome 6 has a regulatory sequence, which acts as a TNF-α repressor site, and the presence of the A allele at position -238 may disrupt this regulatory effect leading to increased transcription of TNF-α. On the other hand, another study showed that peripheral blood mononuclear cells from subjects carrying the TNF-α -238A allele produced less TNF-α after stimulation in contrast to the TNF-α -238G variant.[26] However, other studies reported that -238A variant had no effect on TNF-α production.[21,22] It is difficult to compare TNF-α serum levels in different studies due to their methodological differences.[4] In addition, TNF-α serum levels vary with age, body mass index, gender, time of the day, medicine intake, and several other factors.[20,27] Considering all these factors that affect TNF serum levels, it is possible that the presence of a polymorphism per se may not be sufficient to explain TNF serum levels, which are the result of a complex group of interconnected genetic and environmental factors.[4]

In conclusion, our work showed that TNF-α -238 and -308 polymorphisms were associated with susceptibility to RHD and increased production of TNF-α. Both polymorphisms were related to valvular damage and a more severe outcome of RHD in Egyptian children.

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