Yonsei Medical Journal
Vol. 44, No. 6, pp. 1021 - 1026, 2003

# Tumor Necrosis Factor-$\alpha$ Levels and Promoter Polymorphism in Patients with Kawasaki Disease in Korea

**Sun-Young Ahn[1], Gwang-Cheon Jang[1], Jeon-Soo Shin[2], Kyung Mi Shin[1], and Dong-Soo Kim[1]**

*Departments of [1]Pediatrics and [2]Microbiology, Yonsei University College of Medicine, Seoul, Korea.*

Tumor necrosis factor (TNF)-$\alpha$ plays a major role in the pathogenesis of Kawasaki disease (KD), a systemic vasculitis primarily affecting young children. We performed this study to examine the serum levels of TNF-$\alpha$ and to investigate a possible relation to promoter polymorphism at positions -238 and -308 in KD patients in Korea. We obtained 48 paired serum samples from 24 patients in the acute and subacute stages of KD, and control sera from 12 age-matched children who were having routine blood samples taken before elective surgical procedures.

Our studies showed a significant increase in serum levels of TNF-$\alpha$ measured in the acute stage of KD (24.1 $\pm$ 9.4 pg/mL) compared to those in the subacute stage (11.8 $\pm$ 5.8 pg/mL; $p<0.01$) and normal controls (10.4 $\pm$ 4.9 pg/mL; $p<0.01$). Previous studies report that the presence of the A allele at positions -308 and -238 may be associated with higher TNF-$\alpha$ levels. However, our results showed that the frequency of the A allele at position -308 in the KD patients was the same as the controls (2 out of 24, 8.3% vs. 8.3%, odds ratio (OR)= 1.00), while the frequency of the A allele at position -238 in the KD patients was lower than the controls (0/24, 0% vs. 8.3%, OR=0.00); this difference though was not statistically significant. We concluded that although TNF-$\alpha$ levels were significantly elevated in the acute stage of KD, there was no significant difference in the frequency of the A allele at positions -238 and -308 between the KD and control groups in Korean patients.

**Key Words:** Kawasaki disease, TNF-$\alpha$, polymorphism

## INTRODUCTION

Kawasaki disease (KD) is an acute febrile disease of unknown etiology that occurs primarily in infants and young children. KD involves an immune-mediated inflammatory process that leads to endothelial cell damage, and if left untreated, may result in potentially fatal coronary artery aneurysms. The immunoregulatory changes in KD are evidenced by changes in B- and T-cell levels and an increase in serum levels of cytokines, such as tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1, IL-2, IL-6, and interferon (IFN)-$\gamma$.[1-3]

A possible genetic predisposition for KD has been indicated by its higher incidence among Asians; however, the genetic factors that may lead to an increased susceptibility for KD are still unclear. There have been attempts to find a link between genetic polymorphisms and the expression of cytokines in KD patients. Recent studies have especially focused on the TNF-$\alpha$ gene and the association between genetic polymorphisms in the promoter region and its production.

TNF-$\alpha$ is a proinflammatory cytokine that acts as a potent immune mediator in many infectious and autoimmune diseases. Many polymorphisms, including microsatellites and single nucleotide polymorphisms (SNPs), are present in the TNF gene cluster. These polymorphisms have been associated with a wide range of both infectious and autoimmune diseases, including cerebral malaria and systemic lupus erythematosus (SLE).[4,5] The SNPs include those at positions -1031(T→C), -863(C→A), -851(C →T), -419(G→C), -376(G→A), -308(G→A), and -238(G→A). Among these polymorphisms, the -308 G/A polymorphism has been most extensively studied. Several studies report conflicting results on the effect of the -308 G/A polymorphism on TNF-$\alpha$ production. One study showed that the presence of the A allele at the

Received July 30, 2003
Accepted October 2, 2003

*Reprint address: requests to Dr. Dong-Soo Kim, Department of Pediatrics, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-5524, Fax: 82-2-393-9118, E-mail: dskim6634@yumc.yonsei.ac.kr*

-308 site resulted in increased TNF-α production in stimulated whole blood cell cultures.[6] However, other studies have shown that this polymorphism does not affect TNF-α production.[7] Similar results have been reported for the polymorphism at position -238, including a study showing that the presence of the A allele at -238 did not result in increased TNF-α production.[8]

TNF-α levels increase significantly in the acute phase of KD, suggesting that it may play a major role in the pathogenesis of the vasculitis in KD.[9] Since TNF-α promoter polymorphisms have been associated with various autoimmune and infectious diseases, we studied the possible genetic influence on the expression of TNF-α in KD patients in Korea by examining the serum levels of TNF-α in KD patients and the promoter polymorphisms at positions -238 and -308.

## MATERIALS AND METHODS

### Subjects

Forty-eight paired serum samples were obtained at the acute and subacute stages from 24 patients with KD, 7 days after intravenous injection of immunoglobulin. All these patients were admitted to the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. All patients fulfilled at least five of the six criteria for the diagnosis of KD. Atypical KD was excluded from this study. The control sera were obtained from 12 age-matched children who were having routine blood samples taken before elective surgical procedures. The mean age ± standard deviation (SD) of patients and control groups was 2.1 ± 0.7 years and 2.7 ± 0.9 years respectively. There was no difference in the age and sex ratio between the patients and the control groups. Informed consent was obtained from the parents of each child included in the study.

### TNF-α levels by enzyme-linked immunosorbent assay (ELISA)

All sera were stored at -70℃ until levels of TNF-α were measured using an ELISA kit (Endogen, Woburn, MA, U.S.A). ELISA was performed according to the manufacturer's instructions. Briefly, 100 μl of each patient's serum was put into each microwell of the kit and incubated for 1 hour at 37℃. Positive and negative sera which were supplied in the kit were also added at the same time. The wells were washed three times, and then 100 μl of enzyme-labelled anti-TNF-α conjugates were added and incubated for 1 hour at 37℃. 100 μl of substrate solution was then added and incubated for 15 minutes at 20-25℃ after washing three times. The absorbance was measured at 450 nm after adding 25 μl of stopping solution. All samples were tested in duplicate.

### TNF-a promoter polymorphisms

TNF-α promoter polymorphisms at positions -308 and -238 in KD patients were identified using ABI Prism( SNaPshotTM ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA) with some modification. Briefly, the genomic DNA was extracted from the peripheral blood mononuclear cells of the subjects using QIAamp DNA Blood MiniKit (QIAGEN Inc., Valencia, CA, USA) and then a 328 bp fragment spanning positions -396 to -69 of the 5′ flanking region of the TNF-α gene was amplified using primers TNF-396 (5′-TTCCTGCATCCTGTCTGGAA-3′) and TNF-69 (5′-CAGCGGAAAACTTCCTTGGT-3′).[10,11] PCR was performed in a 50 μL reaction containing 100 ng genomic DNA, 400 μM dNTPs, 10 pM of each primer, and 2.5 U Taq polymerase (TaKaRa, Shiga, Japan). PCR conditions were 94℃ for 60 sec, 55℃ for 60 sec, and 72℃ for 60 sec. After 35 cycles of amplification, an additional extension was conducted at 72℃ for 10 min. PCR products were purified by QIA quick PCR purification kit (QIAGEN) and finally dissolved in 15 μL to 30 μL depending on the PCR band intensity. PCR products were treated with shrimp alkaline phosphatase (SAP; USB Co., Cleveland, OH, USA) to remove PCR primers, which may cause unexpected peaks in electropherograms. After deactivation of SAP at 72℃ for 15 min, SNaPshot ddNTP primer extension reaction was performed. Multiplex PCR was performed in a 10 μL reaction containing 5 μL SNaPshot Ready Reaction Premix (Applied Biosystems), 2 μL of a mixture of four primers containing 0.25 pmol of each primer, and

0.15 pmol of template DNA. The primers were TNFA308-sense (5′-AATAGGTTTTGAGGGGC ATG-3′) and TNFA308-antisense (5′-TTTTTTCTGGA GGCTGAACCCCGTCC-3′) for position -308, and TNF A238-sense (5′-TTTTTTTTTTTTTTTTAGAA GACCCCCCTCGGAATC-3′) and TNFA238-antisense (5′-TTTTTTTTTTTTTTTTTTTTTTACTCCCC ATCCTCCCTGCTC-3′) for position -238. PCR conditions were 96℃ for 10 sec, 50℃ for 5 sec, and 60℃ for 30 sec. After 25 cycles, the PCR reaction mixture was incubated with 0.5 unit SAP for 1 h at 37℃, and then SAP activity was deactivated. One μL of sample was mixed with 20μL of formamide and denatured for 5 min at 95℃. The samples were loaded on ABI Prism 310 Genetic Analyzer (Applied Biosystems) for the data analysis.

### Statistical analysis

A paired Student's t-test was used to compare the paired levels of TNF-α in the serum samples of the patients in the acute and subacute phases. The Wilcoxon-signed rank sum test was used to compare the values of the TNF-α levels between the groups of patients in the acute phase of KD and the control groups. A $p$ value of less than 0.01 was considered statistically significant.

The statistical significance of any difference in frequency of each polymorphic allele or genotype between the patient and the control group was evaluated by the Chi-squared test. $P$ values were calculated using Fisher's exact probability ($p<0.01$ was considered statistically significant). Odds ratio (OR) was calculated as a measurement of strength of association according to Woolf's method.[12]

## RESULTS

### Serum TNF-α levels

We measured the serum TNF-α levels in the acute and the subacute phases of KD in 24 patients. The serum TNF-α level was 24.1 ± 9.4 pg/mL in the acute phase, higher than in the subacute phase (11.8 ± 5.8 pg/mL, $p<0.01$) and the controls (10.4 ± 4.9 pg/mL, $p<0.01$). After the intravenous administration of immunoglobulin, we found that serum TNF-α levels decreased to near normal levels in the subacute phase; therefore, there was no significant difference between the controls and the subacute phase of disease (Fig. 1). Except for two patients, all patients showed a decrease in serum TNF-α levels during the subacute phase of disease (Fig. 2).

### Allele frequencies of the promoter region of the TNF-α gene in Kawasaki disease patients

The frequency of the A allele at position -308 in the KD patients was the same as the controls (2 out of 24, 8.3% vs. 8.3%, OR=1.00). The frequency of the A allele at position -238 in the controls (8.3% vs. 0%, OR=0.00) was slightly greater than in the KD patients, however the difference was not statistically significant (Table 1).

## DISCUSSION

The etiology for KD remains unknown, but

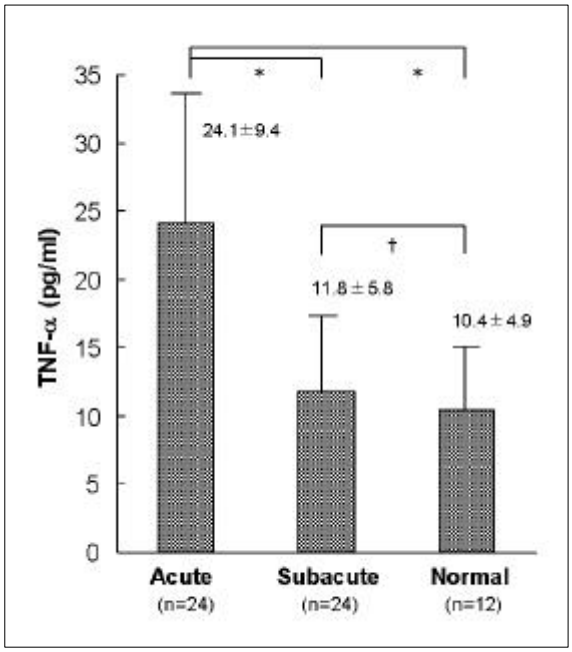


**Fig. 1.** Serum levels of TNF-α in Kawasaki patients. The levels of TNF-α were measured by ELISA in the patients with acute and subacute stages of Kawasaki disease and normal controls. *$p<0.01$ compared to subacute stage and normal controls. †$p>0.05$ compared to normal controls.

several studies have suggested the involvement of an immune-mediated process. Cytokines such as IL-1, IL-6, IFN-α and TNF-α are released in KD,[1-3] inducing a series of inflammatory reactions that lead to increased levels of peripheral blood T cells, B cells, and macrophages. These changes lead to endothelial cell injury and inflammation of small and medium-sized arteries, especially coronary arteries. TNF-α plays a major role in this inflammatory process.[9]

TNF-α is a proinflammatory cytokine that has been implicated in the pathogenesis of a wide range of diseases. The TNF-α gene is located within the class III region of the highly polymorphic major histocompatibility (MHC) complex on human chromosome 6p21. This genetic location and the association of certain MHC haplotypes with different TNF-α phenotypes[13] suggest that polymorphisms within the TNF-α gene may contribute to TNF-α production and its role in various autoimmune and infectious diseases. Peripheral blood mononuclear cells with polymorphisms at -1031C, -863A, or -857T showed an increase in TNF-α production when treated with concanavalin-A indicating that polymorphisms in the promoter region of TNF-α regulate TNF-α levels.[14] Several studies have also shown an association between polymorphisms of the TNF-α gene and a wide range of diseases such as cerebral malaria and chronic active hepatitis C infection.[4,15]

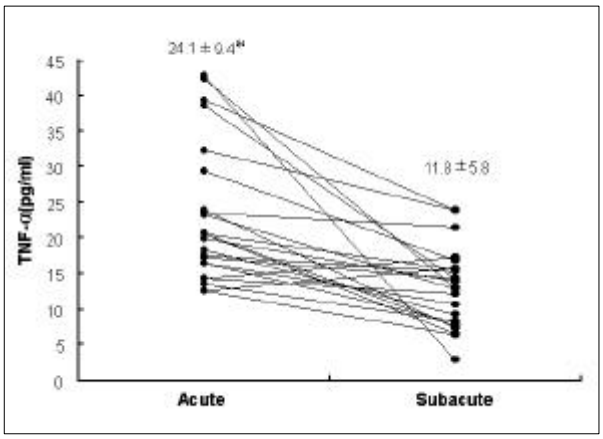


**Fig. 2.** Serum levels of TNF-α in serial samples from Kawasaki patients. The levels of TNF-α were measured by ELISA in serial samples obtained from patients during acute stage of Kawasaki disease and subsequently during subacute stage of their illness. *$p<0.01$ compared to subacute stage.

Other polymorphisms within the TNF-α gene shown to be associated with autoimmune and infectious diseases include polymorphisms at positions -308 and -238, involving the substitution of guanine by adenosine. The presence of the A allele at position -308 has especially been shown to be a strong transcriptional activator of TNF-α.[16] This allele has been associated with an increased risk for cerebral malaria.[4] The -238A allele has been associated with chronic hepatitis B[10] and hepatitis C.[15]

In this study, we observed a significant increase in serum levels of TNF-α in the acute stage of KD (24.1 ± 9.4 pg/mL) compared to those in the subacute stage (11.8 ± 5.8 pg/mL, $p<0.01$) and normal controls (10.4 ± 4.9 pg/mL, $p<0.01$). Although we expected to find a relation between TNF-α polymorphisms and KD, our results showed that the frequency of the A allele at position -308 in the KD patients (8.3% vs. 8.3%, OR=1.00) did not differ significantly from the controls. The frequency of the A allele at position -238 in the controls (8.3% vs. 0%, OR=0.00) was slightly greater than

**Table 1.** Genotypes and Allele Frequency of Polymorphisms at Positions -308 and -238 in the TNF-α Gene

| | Control (n=12) | Kawasaki patients (n=24) |
|---|---|---|
| TNF-α -308* | | |
| G/G | 11/12 (91.7) | 22/24 (91.7) |
| G/A | 1/12 (8.3) | 2/24 (8.3) |
| O.R. | - | 1.0 |
| TNF-α -238 | | |
| G/G | 11/12 (91.7) | 24/24 (100) |
| G/A | 1/12 (8.3) | 0/24 (0) |
| O.R.† | - | 0.0 |

*tumor necrosis factor-α, †odds ratio.

in the KD patients, however the difference was not statistically significant.

Several studies have shown conflicting results on the genetic influence in KD. HLA-Bw22 has been associated with KD patients in Japan.[17] On the other hand, another study showed no association between MHC class II alleles in the pathogenesis of KD.[18] Recent studies investigating the association between genetic polymorphisms within the TNF-α promoter and TNF-α levels in KD patients have also been reported. One study showed that the TNF-α -308 A/G genotype was found at a higher frequency among white children with KD who had coronary artery abnormalities compared with controls.[19] However, another study showed no association between the levels of TNF-α production and genetic polymorphisms in the 5′ flanking region of the TNF-α gene in Japanese children with a history of KD,[20] which was consistent with the results of our study. Although several studies support the role of promoter polymorphisms in increased TNF-α production, other studies have shown that these polymorphisms may not affect production. Pociot et al. reported that TNF-α production did not differ significantly in monocytes stimulated *in vitro* with lipopolysaccharides from TNFA -238 G/G homozygous and TNFA -238 A/G heterozygous individuals.[8] This finding that promoter polymorphisms may not affect TNF-α production may explain the results of our study. However, another explanation may be that TNF-α production, which is regulated at both the transcriptional and posttranscriptional level, is controlled by multiple genetic factors interacting together and thus polymorphisms at positions -308 and -238 must be present with other polymorphisms or genetic elements to control TNF-α production. The specific genetic mechanism involved in the regulation of TNF-α production remains to be elucidated.

In summary, our study has shown a significant increase in the TNF-α levels in the acute phase of KD compared to those in the subacute phase and controls. However, we found no significant difference in the frequency of the A allele at positions -308 and -238 of the TNF-α gene between KD and normal control groups in Korean patients. A limitation of our study was the relatively small sample size. Further studies could include investigations of other loci in the promoter region and their corresponding influence on TNF-α levels. Cardiac involvement is a serious cause of morbidity in KD and since genetic factors may affect the degree of inflammatory response, identifying these factors may contribute to predicting the susceptibility to coronary artery disease in KD.

## REFERENCES


1. Leung DYM, Geha RS, Newberger JW, Burns JC, Fiers W, Lapierre LA, et al. Two monokines, interleukin-1 and tumor necrosis factor, render cultured vascular endothelial cells susceptible to lysis by antibodies circulating during Kawasaki syndrome. J Exp Med 1986; 164:1958-72.
2. Lin CY, Lin CC, Hwang B, Chiang BN. The changes of interleukin-2, tumor necrotic factor and gamma-interferon production among patients with Kawasaki disease. Eur J Pediatr 1991;41:179-82.
3. Kim DS. Serum IL-6 in Kawasaki disease. Yonsei Med J 1992;32:183-8.
4. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature 1994;371:508-10.
5. Wilson AG, Gordon C, diGiovine FS, deVries N, van de Putte LB, Emery P, et al. A genetic association between systemic lupus erythematosus and tumor necrosis factor alpha. Eur J Immunol 1994;24:191-5.
6. Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S, et al. Tumour necrosis factor (TNF) gene polymorphism influences. TNF-α production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol 1998;113:401-6.
7. Brinkman BM, Zuijdeest D, Kaijzel EL, Breedveld FC, Verweij CL. Relevance of the tumor necrosis factor alpha -308 promoter polymorphism in TNF alpha gene regulation. J Inflamm 1995-1996;46:32-41.
8. Pociot F, D'Alfonso S, Compasso S, Scorza R, Richiardi PM. Functional analysis of a new polymorphism in the human TNF alpha gene promoter. Scand J Immunol 1995;42:501-4.
9. Furukawa S, Matsubara T, Jujoh K, Yone K, Sugawara T, Sasai K, et al. Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. Clin Immunol Immunopathol 1988;48:247-51.
10. Hohler T, Kruger A, Gerken G, Schneider PM, Meyer zum Buschenefelde KH, Rittner C. A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. Clin Exp Immunol 1998;111:579-82.
11. Turner D, Choudhury F, Reynard M, Railton D, Navarrete C. Typing of multiple single nucleotide

polymorphisms in cytokine and receptor genes using sNaPshot. Hum Immunol 2002;63:508-13.
12. Woolf B. On estimating the relation between blood group and disease. Ann Hum Genet 1955;41:251-3.
13. Abraham LJ, French MA, Dawkins RL. Polymorphic MHC ancestral haplotypes affect the activity of tumor necrosis factor-alpha. Clin Exp Immunol 1993;92:14-8.
14. Higuchi T, Seki N, Kamizono S, Yamada A, Kimura A, Kato H, et al. Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens 1998;51:605-12.
15. Hohler T, Kruger A, Gerken G, Schneider PM, Meyer zum Buschenefelde KH, Rittner C. Tumor necrosis factor alpha promoter polymorphism at position -238 is associated with chronic active hepatitis C infection. J Med Virol 1998;54:173-7.
16. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor-$\alpha$ promoter on transcription activation. Proc Natl Acad Sci USA 1997;94:3195-9.
17. Kato S, Kimura M, Tsuji K, Kusakawa S, Asai T, Juji T, et al. HLA antigens in Kawasaki disease. Pediatrics 1978;61:252-5.
18. Barron KS, Silverman ED, Gonzales JC, St Clair M, Anderson K, Reveille JD. Major histocompatibility complex class II alleles in Kawasaki syndrome - lack of consistent correlation with disease or cardiac involvement. J Rheumatol 1992;19:1790-3.
19. Quasney MW, Bronstein DE, Cantor RM, Zhang Q, Stroupe C, Shike H, et al. Increased frequency of alleles associated with elevated tumor necrosis factor-$\alpha$ levels in children with Kawasaki disease. Pediatr Res 2001; 49:686-90.
20. Kamizono S, Yamada A, Higuchi T, Kato H, Itoh K. Analysis of tumor necrosis factor-$\alpha$ production and polymorphisms of the tumor necrosis factor-$\alpha$ gene in individuals with a history of Kawasaki disease. Pediatr Int 1999;41:341-5.