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Is Tumor Necrosis Factor Genotype (*TNFA2/TNFA2*) a Genetic Prognostic Factor of an Unfavorable Outcome in IgA Nephropathy?

The purpose of this study was to examine whether there are the associations between TNF α and TNF β gene polymorphisms and the development and progression of Ig A nephropathy (IgAN). A cross-sectional study on *TNF* α and *TNF* β gene polymorphisms by polymerase chain reaction with restriction fragment length polymorphisms was performed on 76 patients with primary IgAN confirmed by renal biopsy and 100 healthy controls. The allele with G→A substitution was designated as *TNFA2* for the *TNF* α gene and *TNFB2* for the *TNF* β gene. A patient in whom dialysis treatment was started or whose serum creatinine became double or over during the follow-up duration was designated as a "progressor". The *TNFA2/TNFA2* genotype was more prevalent in the progressor than in the non-pregressor group (20.0 vs 3.3%, $p<0.05$). Clinical factors such as serum creatinine, systolic and diastolic blood pressure ($p<0.001$, respectively) were higher and pathologic factor such as Grade IV or V renal lesions was more prevalent ($p<0.01$) in the progressor than in the non-progressor group. Therefore, *TNFA2/TNFA2* genotype may be a risk factor for the progression of IgAN.

Key Words : *Glomerulonephritis, IgA; Tumor Necrosis Factor; Polymorphism (Genetics)*

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INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis and is an important cause of end-stage renal disease requiring renal replacement therapy (1). Although the pathogenetic mechanism of IgAN is still controversial, a great body of evidence suggests that cytokines such as tumor necrosis factor (TNF) and interleukin play an important role in the renal injury of IgAN (2, 3).

TNF α and TNF β are potent cytokines which are associated with the inflammatory response (4). TNF is involved in the initiation or progression of renal injury (5). Increased expression of TNF has been demonstrated in vivo in several animal models of glomerulonephritis (6). *TNF* α and *TNF* β genes are closely linked and tandemly arranged in the human genome and map between class I and class III regions of major histocompatibility complex (MHC) in humans (4). The location of *TNF* genes within the MHC raised a possibility that some MHC-linked inflammatory diseases might be related to genetic variations inside the *TNF* locus. A genomic polymorphism within the *TNF* locus influences plasma TNF concentration (7). It is known that there are at least two polymorphisms in the class III region controlling TNF α production. One is at position -308 base pairs in the promoter region of the *TNF* α gene (7), for which allele 2 (*TNF2*) is associated with higher constitutive and inducible levels of TNF α (8). The other is in intron 2/exon 3 of the *TNF* gene, with the two polymorphic alleles variably associated with high and low levels of TNF α secretion by mononuclear cells, depending on the population under investigation (9, 10). However, the role of *TNF* gene polymorphism in IgAN is not clear as yet.

We evaluated the G→A transition polymorphism of the *TNF* α and *TNF* β gene with *Nco*I restriction fragment length analysis and their clinical significances in seventy-six patients with primary IgAN confirmed by renal biopsy.

MATERIALS AND METHODS

A total of 76 patients (38 males, 38 females; mean age, 30.4 yr) diagnosed as IgA nephropathy by renal biopsy were included in this study. We excluded patients with glomerulonephritis associated with diabetes mellitus, systemic lupus erythematosus, or hepatitis B or C virus. One hundred healthy volunteers (49 males, 51 females; mean age, 48.2 yr) were recruited during an annual physical examination at Soonchunhyang Chonan Hospital (Chonan, Korea) as normal controls.

Genomic DNA was extracted by DNA extraction column (QIAmp blood kit; Qiagen, Venlo, The Netherlands) from

peripheral blood. Polymerase chain reaction (PCR) was performed using the primer sets under the conditions previously described (7, 11): sense primers (*TNFα*: 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′; *TNFβ*: 5′-CCG TGC TTC GTG CTT TGG ACT A-3′), antisense primers (*TNFα*: 5′-TCC TCC CTG CTC CGA TTC CG-3′; *TNFβ*: 5′-AGA GGG GTG GAT GCT TGG GTT C-3′), and a GeneAmp PCR machine (Perkin Elmer 9600; Perkin Elmer, Norwalk, CT) were used. Products of 107 and 782 bp were generated for *TNFA* and *TNFB*, respectively. The amplified PCR products were digested with *Nco*I and analysed by gel electrophoresis. For *TNFα*, primers were designated to incorporate a polymorphic site at a position -308 bp of the *TNFα* gene into a *Nco*I restriction site. Restriction digests generated products of 87 and 20 bp for allele 1 (*TNFA1*) and 107 bp for allele 2 (*TNFA2*). The 782 bp fragment amplified across intron 2 of the *TNFβ* gene also incorporates a polymorphic *Nco*I site, which generates fragments of 782 bp for *TNFB2* and 586 plus 196 bp for *TNFB1*. Ethidium bromide staining of the gel demonstrated the original 782 bp fragment (homozygous patients for allele *TNFB2*, lacking the *Nco*I site), three fragments of 782, 586, and 196 bp of length (heterozygous patients), or two fragments of 586 and 196 bp of size (homozygous patients for the allele *TNFB1*) (Fig. 1). The clinical parameters at renal biopsy and the latest follow-up data were collected. A patient in whom dialysis treatment was started or whose serum creatinine became double or over during the follow-up duration was designated as "progressor".

Data were expressed as mean±SD for the age, follow-up duration, 24-hr proteinuria, serum creatinine, systolic and diastolic blood pressures, hematocrit, and cholesterol. Statistical analyses of genotype distributions and allele frequencies were performed by an analysis of variance test or χ^2 test. *p*-value was calculated by the Student's t-test for continuous variables or Fisher's exact test for categorical variables. $p<0.05$ was considered to be statistically significant's.

Table 1. Distribution of tumor necrosis factor (*TNF*) α and *TNFβ* gene frequencies in control subjects and patients with IgA nephropathy

			Control	IgA nephropathy
TNF α	Genotype	*TNFA1/TNFA1*	65 (65.0)	45 (59.2)
		TNFA1/TNFA2	31 (31.0)	26 (34.2)
		TNFA2/TNFA2	4 (4.0)	5 (6.6)
	Allele	*TNFA1*	161 (80.5)	116 (76.3)
		TNFA2	39 (19.5)	36 (23.7)
TNF β	Genotype	*TNFB1/TNFB1*	4 (4.0)	8 (10.5)
		TNFB1/TNFB2	57 (57.0)	37 (48.7)
		TNFB2/TNFB2	39 (39.0)	31 (40.8)
	Allele	*TNFB1*	65 (32.5)	53 (34.9)
		TNFB2	135 (67.5)	99 (65.1)

Values are expressed as number (percent). There were no significant statistical differences between the groups.

RESULTS

Polymorphism of the *TNFα* gene

The genotype of *TNFα* distribution was as follows: in the 76 patients with IgAN, 45 patients (59.2%) had the *TNFA1/TNFA1* genotype, 26 patients (34.2%) had the *TNFA1/TNFA2* genotype, and 5 patients (6.6%) had the *TNFA2/TNFA2* genotype. There was no significant difference in the distribution of genotype or allele frequencies of the *TNFα* gene between IgAN patients and control subjects (Table 1). There was no significant difference in clinical parameters which included the age, 24-hr proteinuria, serum creatinine, hematocrit, cholesterol, and systolic and diastolic BP at renal biopsy among the three *TNFα* genotypes. And neither was there any difference in the follow-up duration of the three

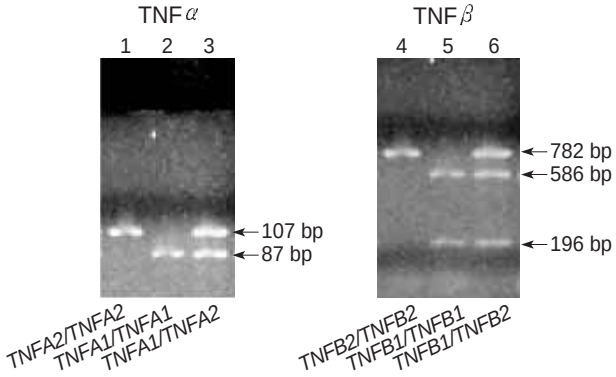

Fig. 1. Electrophoresis of the PCR product of the TNF α and TNF β genes. Lane 1 is a homozygote of *TNFA2*, lane 2 is a homozygote of *TNFA1*, and lane 3 is a heterozygote of *TNFA1* and *TNFA2*. Lane 4 is a homozygote of *TNFB2*, lane 5 is a homozygote of *TNFB1*, and lane 6 is a heterozygote of *TNFA1* and *TNFA2*.

Table 2. Comparison of clinical characteristics in patients with IgA nephropathy at renal biopsy and the lastest follow-up by *TNFα* genotypes

	TNFA1/TNFA1 (n=45)	*TNFA1/TNFA2* (n=26)	*TNFA2/TNFA2* (n=5)
At renal biopsy			
Age (yr)	31.4±10.2	31.9±10.2	36.8±10.7
Proteinuria (mg/24 hr)	2,428±2,737	3,164±3,519	5,240±3,150
Serum creatinine (mg/dL)	1.2±0.5	1.1±0.4	1.2±0.6
Systolic BP (mmHg)	131.2±22.6	138.1±18.8	144±18.1
Diastolic BP (mmHg)	83.8±15.3	85.8±12.1	94±15.2
Hematocrit (%)	40.0±5.3	40.0±4.2	43.8±7.5
Cholesterol (mg/dL)	216.7±54.9	202.1±49.1	199.2±52.2
At follow-up			
Progressor (%)	22.2	7.7*	60.0*

Values are expressed as mean±S.D. or percent. *p*-value was calculated by ANOVA for continuous variables or Fisher's exact test for categorical variables. *$p<0.05$. A patient in whom dialysis treatment was started or whose serum creatinine became double or over during the follow-up duration was designated as "progressor".

Table 3. Comparison of clinical characteristics in patients with IgA nephropathy at renal biopsy and the lastest follow-up by *TNFβ* genotypes

	TNFB1/TNFB1 (n=8)	*TNFB1/TNFB2* (n=37)	*TNFB2/TNFB2* (n=31)
At renal biopsy			
Age (yr)	32.1±10.4	30.6±10.7	32.0±8.4
Proteinuria (mg/24 hr)	3,000±2,384	3,137±3,696	2,529±2,415
Serum creatinine (mg/dL)	1.44±0.83	1.07±0.40	1.1±0.38
Systolic BP (mmHg)	143.3±25.0	131.7±20.4	136.6±21.3
Diastolic BP (mmHg)	86.7±15.1	84.7±12.8	85.5±16.2
Hematocrit (%)	40.6±4.2	38.9±4.6	42.0±5.5
Cholesterol (mg/dL)	223.3±43.9	213.7±55.0	201.7±51.4
At follow-up			
Progressor (%)	25.0	10.8	29.0

Values are expressed as mean±S.D. or percent. Statistical difference was calculated by ANOVA for continuous variables or Fisher's exact test for categorical variables. There were no significant statistical differences among the groups. A patient in whom dialysis treatment was started or whose serum creatinine became double or over during the follow-up duration was designated as "progressor".

TNFα genotypes (55.3±40.6 vs 65.8±34.1 vs 71.2±35.4 months). However, the prevalence of a progressor among the three genotypes was significantly different (*TNFA2/TNFA2* vs *TNFA1/TNFA2,* 60.0 vs 7.7%, $p<0.05$, Table 2).

Polymorphism of the *TNFβ* gene

The genotype of TNFβ distribution were as follows: in the patients with IgAN, 8 patients (10.5%) had the *TNFB1/TNFB1* genotype, 37 patients (48.7%) had the *TNFB1/TNFB2* genotype, and 31 patients (40.8%) had the *TNFB2/TNFB2* genotype. There was no significant difference in the distribution of the genotype of the *TNFβ* gene between the IgAN and control subjects (Table 1). The overall allele frequency of TNFβ was also shown in Table 1. There were no significant differences in the clinical parameters which included the age, 24-hr proteinuria, serum creatinine, hematocrit, cholesterol, systolic and diastolic BP at renal biopsy (Table 3), follow-up duration and the frequency of those belonging to the progressor group among the three *TNFβ* genotypes (52.0±27.4 vs 57.8±37.9 vs 64.6±41.2 months).

Prognostic factors of progression of IgAN

The follow-up duration was not significantly different between the non-progressor and progressor groups (54.8±36.9 vs 80.0±37.3 months). The systolic and diastolic blood pressure at renal biopsy were significant higher in the progressor group than non-progressor group ($p<0.001$, respectively, Table 4). The proteinuria at renal biopsy was higher in the progressor group than non-progressor group, however, it did not reach the statistical significance (Table 4). There were

Table 4. Clinical predictors of progression of renal failure in patients with IgA nephropathy

	Non-progressor (n=61)	Progressor (n=15)
Clinical Factors		
Age (yr)	30.0±8.1	33.0±8.5
Proteinuria (mg/24 hr)	2,497±3,162	4,179±2,894
Serum creatinine (mg/dL)	1.02±0.34	1.53±0.56‡
Systolic BP (mmHg)	131.5±19.7	156.7±20.6‡
Diastolic BP (mmHg)	81.9±12.7	101.1±16.2‡
Hematocrit (%)	40.1±4.9	42.5±7.8
Cholesterol (mg/dL)	207.0±53.7	219.7±40.8
Rate of decline of renal function	0.155±0.356	0.796±0.419‡
Pathologic Factor		
Class IV or V lesions (%)+	12.2	50.0†
Genetic Factor		
TNFA2/TNFA2 genotype (%)	3.3	20.0*

Values are expressed as mean±S.D. or percent. Rate of decline of renal function was calculated as [(1/serum creatinine at renal biopsy-1/serum creatinine at the latest follow-up)/months of follow-up duration] ×100. +Grading system according to Lee (12) was available only for 49 patients due to insufficient kidney biopsy material. *p*-value was calculated by Student's t-test for continuous variables or Fisher's exact test for categorical variables, *$p<0.05$, †$p<0.01$, ‡$p<0.001$.

no significant differences in the age, hematocrit, and cholesterol at renal biopsy between the groups (Table 4). The prevalence of Grade IV or V renal lesions (12) was higher in the progressor group than non-progressor group ($p<0.01$, Table 4). The serum creatinines at renal biopsy ($p<0.001$, Table 4) and at the latest follow-up (1.28±1.13 vs 6.09±2.11, $p<0.001$) were higher in the progressor group than in the non-progressive group. The rate of decline of renal function was calculated as [(1/serum creatinine at renal biopsy-1/serum creatinine at the latest follow-up)/months of follow-up duration] ×100. It was significantly higher in the progressor group than non-progressor group ($p<0.001$, Table 4). In the progressor group, 12 patients with end-stage renal disease were in-

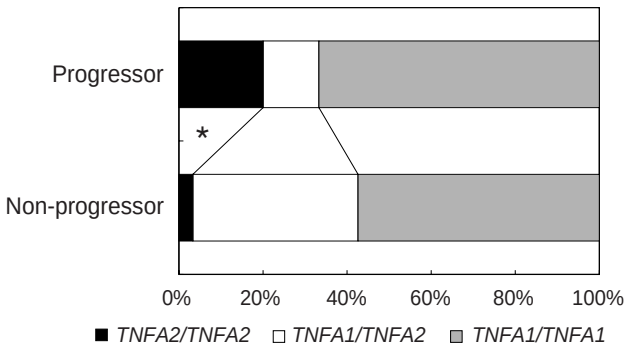

Fig. 2. Distribution of *TNFα* genotypes in the patients with IgA nephropathy. A progressor means a patient whose serum creatinine became double or over during the follow-up duration more than three years. *TNFA2/TNFA2* genotype was more prevalent in the progressor group ($p<0.05$).

cluded. Nine patients had the *TNFA1/TNFA1* genotype, 2 patients had the *TNFA1/ TNFA2* genotype, and 1 patient had the TNFA2/TNFA2 genotype. The *TNFA2/TNFA2* genotype was more prevalent in the progressor group than non-progressor group ($p<0.05$, Table 4, Fig. 2).

DISCUSSION

TNFα is a proinflammatory cytokine that has been involved in certain forms of immune-mediated renal injury, including IgAN (5). TNFα is synthesized by glomerular cells or circulating macrophage/monocytes (13, 14). TNF activates mesangial cells to release procoagulants, plasminogen activator, and plasminogen activator inhibitor (13). Increased expression of TNF has been demonstrated in vivo in several animal models of glomerulonephritis including murine lupus models (6). The administration of TNF potentiates renal injury in IgAN, murine lupus models, and anti-GBM antibody disease. The location of *TNF* genes within the MHC raised a possibility that some MHC-linked inflammatory diseases might be related to genetic variations inside the *TNF* locus. The polymorphism of gene encoding TNFα at position -308 in the promoter region involving a transition of guanidine to adenosine has been characterized (11). The -308A polymorphism (*TNFA2*) is associated with an increase of *TNFα* transcription (15), which may predispose humans to the occurrence of inflammatory diseases. Carriage of the *TNFA2* allele has been shown to be associated with several inflammatory diseases (16). There was no significant difference in the distribution of genotype or allele frequencies of the *TNFα* gene between IgAN patients and control subjects in this study. Our result was similar to previous reports (17). Therefore, we speculate that the *TNFα* gene polymorphism is not associated with the development of IgAN.

The clinical course of IgAN is extremely variable. The clinical predictors including impairment of renal function, severe proteinuria, and arterial hypertension at presentation have been known as of an unfavorable outcome of IgAN (18). Our data supported these previous observations. The clinical parameters including serum creatinine, systolic and diastolic blood pressure at renal biopsy and the prevalence of Grade IV or V renal pathologic lesions were significantly higher in the progressor group than in the non-progressor group in this study. These also support previous results (18).

Besides clinical and pathologic prognostic factors, the interest to search for genetic markers such as HLA-B35 or specific genotype of angiotensin converting enzyme (*ACE*) polymophism for progression of IgAN has increased. However, no study has confirmed the association between the progression of IgAN and the presence of specific *HLA* or *ACE* genotypes (18). Also, the prognostic significance of *TNFα* gene polymorphism in IgAN patients on the progression of the disease has not been described so far. The patients with IgAN who had a progressive disease were more commonly observed in the subgroup of the *TNFA2/TNFA2* genotype and the *TNFA2/TNFA2* genotype was more prevalent in the progressor group than in the non-progressor group in our study. This is the first report, to our knowledge, that the *TNFA2/TNFA2* genotype is a genetic prognostic factor in IgAN.

There are some limitations in this study. Firstly, the number of patients with *TNFA2/TNFA2* was too small to perform multiple logistic regression analysis. Secondly, we used the changes of 1/(serum creatinine) as the parameter of renal progression of IgAN because other parameters such as creatinine clearance were not available. Thirdly, this was a cross-sectional study, and we obtained the data retrospectively. Therefore, there might have been some bias for the inclusion of patients. Fourthly, all study subjects are Koreans in our study, therefore, we could not rule out possible ethnic differences. Lastly, we did not analyze the influence of various treatments.

The *TNFβ* gene is closely linked to *TNFα*, tandemly arranged in the human genome, and map between class I and class III regions of MHC in humans (4). The two *Nco*I-defined *TNFB* alleles, which differ in structure and in the level of *TNFβ* inducibility, might be relevant for *MHC*-associated predispositions for autoimmune diseases (8). The *TNFB1* allele is strongly linked to the altered production of TNFβ and the variant polypeptide structure might contribute to disease susceptibility of this haplotype. It is conceivable that an allelically varying TNFβ response of activated T lymphocytes might contribute to the inflammatory mechanism of local autoimmune reactions (8). Previous reports demonstrated an association between a *Nco*I *TNFβ* polymorphism and immune-mediated glomerulonephritis including membranous nephropathy and IgAN. They explained this association by linkage disequilibrium with extended *MHC* haplotypes, however, whether the *TNFβ* polymorphism per se is involved in disease susceptibility is not clear in IgAN (10). We could not find any evidence for the association between *TNFβ* polymorphism and IgAN in this study.

In conclusion, this is the first report showing the association between the specific *TNF* genotype and the progression of IgAN. The polymorphisms in the *TNFα* and *TNFβ* gene were not associated with the development of IgAN, however, the *TNFA2/TNFA2* genotype of the *TNFα* gene was more commonly found in patients with IgAN who had a progressive disease than those with the *TNFA1/TNFA1* or *TNFA1/TNFA2* genotype. Therefore, although there were some limitations in this study, it is suggested that the *TNFA2/TNFA2* genotype may be a risk factor for the progression of IgAN.

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