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TNFB Gene Polymorphism in Patients with Systemic Lupus Erythematosus in Korean

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Objectives: *To elucidate the gene frequency of TNFB NcoI polymorphism and its association with HLA class II antigen in patients with systemic lupus erythematosus(SLE) in Korea.*

Methods: *We investigated the gene frequency of the TNFB alleles using DNA obtained from peripheral mononuclar cells in 141 healthy controls and in 58 patients with SLE. The polymorphisms of TNFB gene (735 bp) were studied by NcoI PCR-RELP. A portion of TNFB gene(735 bp) was amplified by PCR and its products were digested with NcoI restriction enzyme. The digested samples of amplified DNA were analyzed by agarose gel electrophoresis. TNFB*1 and TNFB*2 alleles were identified according to polymorphic fragments on NcoI restriction site in the first intron of the TNFB gene. The generic types of HLA-DRBI were also determined by PCR with sequence specific primers(SSP) using genomic DNA from the same subjects.*

Results: *The genotypic frequency of TNFB*2 homozygote was significantly increased in patients with SLE compared with controls(RR=2.36, P=0.011). The frequency of HLA-DRBI*15 was also significantly increased in patients (RR=2.27, P=0.029). However, the increased frequency of TNFB*2 homozygote was apparently increased in nephritis group (RR=2.79, P=0.035), whereas the significance of TNFB*2 homozygote was weakend in non-nephritis group.*

Conclusions: *Our results suggest that genetic predisposition of TNFB*2 homozygote is another risk factor in Korean SLE, especially in DR2 negative patients. In addition, TNFB*2 homozygote could have a tendency for the development of nephritis in patients with SLE.*

Key Words : *SLE, TNFB Gene, HLA-DR, Lupus nephritis, PCR-RFLP*

INTRODUCTION

TNF(tumor necrosis factor) consists of two distinct peptides with multiple immunological and local as well as systemic inflammatory activities. TNF-α, also called cachectin, is produced by activated macrophages and other cells and has a broad spectrum of biological actions on many immune and nonimmune target cells. TNF-β, also called lymphotoxin, is primarliy a product of T lymphocytes and shars many of the biologic activities of TNF-α[1]. TNF-α and TNF-β bind to the same receptor on target cells and are part of a network of interactive signals that orchestrate inflammatory and immunological events[2].

Linkage analysis and pulsed-field gel electrophoresis studies have shown that the gene for TNF-α is linked to the gene for TNF-β and is located between the HLA calss III region and the HLA-B locus in mice and humans[3-5].In systemic lupus erythematosus(SLE), an autoimmune disease characterized by abnormal immune response and various clinical manifestations, the association with the major histocompatibility complex (MHC) class II and III alleles was reported with some ethnic differences[6-8]. In our previous re-

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port[8], HLA-DRBI*1501 and C4AQO allele were associated with susceptibility to Korean SLE. Despite this association, the role of the MHC molecules in SLE remains largely unknown. The locallization of the TNF genes in the MHC, and the link between TNF and regulation of the immune response have led to the hypothesis that polymorphism of TNF gene could be associated with HLA antigens, and might play an important role in the pathogenesis of various HLA associated autoimmune dieseases[3]. Jacob et al[9] reported that TNF-α prodution levles in human. are associated with MHC calss II, and also associated with nephritis in patients with SLE. Two TNFB alleles(TNFB*1 and TNFB*2) distinguished by a polymorphic NcoI restriction site in the first intron have been identified[10]. Some investigators have analyzed the frequency of the NcoI RELP in various autoimmune diseases including SLE[13].The small number of the patients studied and the strong association between HLA and TNF allele have made it difficult to understand the role of TNFB gene polymorphism in human SLE.

Therefore, we have investigated the frequency of the TNFB alleles using PCR-RFLP to determine the role of TNFB gene polymorphism in SLE, and also its genetic relationship to the development of nephritis in patients with SLE.

MATERIALS AND METHODS

Subjects. Fifty-eight Korean patients with SLE (57 women and 1 man) and 141 age mached healthy Koreans were studied. All patients with SLE fulfilled the revised criteria of the American College of Rheumatology (ACR). Careful family history taking exclued autoimmune diseases in controls and their relatives. The patients were divided into nephritis and non-nephritis groups. Diagnosis of lupus nephritis was essentially based on renal biopsy findings or presence of continuous proteinuria(>1 g/24hr), hematuria(>5rbc/HPF), persistent abnormal urine sediments, or renal failure(greather than 30% reduction of Ccr over 1 year), which was not due to another independent cause. Since the risk of acquiring nephritis is the greatest in the first 5 years after SLE diagnosis, patients with the history of recent diagnosis(<5 years) were excluded.

PCR-RFLP for TNFB gene polymorphism. Genomic DNA was extracted from peripheral blood lymphocytes and one microgram was subjected for PCR using 2.5 units of Taq DNA polymerase. TNF5P(5'-CCGTGCTTCGTGCTTT GGA-CTA-3') and TNF3P (5'-AGAGCTGG-TGG-GGACAT-GTCTG-3'), which were previously used for nucleic acid sequencing by Messer et al[14], were used for TNF gene amplification. Amplification was perfomed by 30 cycles of denaturation(94℃), annealing(60℃) and polymerization(72℃). Twenty μl of amplified DNA were directly digested with 2 unit of NcoI for 3 hours at 37℃. The digested samples of amplified DNA were subjected to 1.5% agarose-gel electrophoresis and stained with ethidium bromide. Large(735bp) and small(539bp/196bp) fragments were identified as NcoI site negative (TNFB*2 allele) and positive (TNFB*1 allele), respectively(Fig. 1).

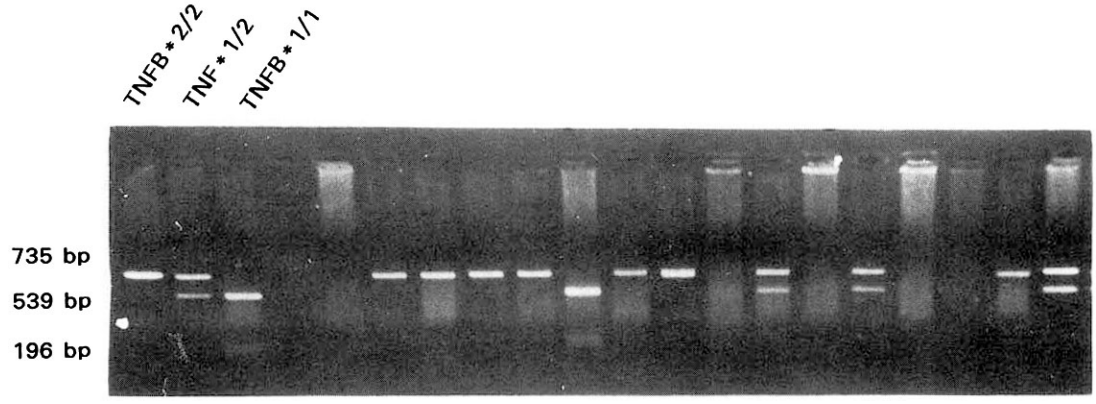

Fig. 1. Patterns of NcoI restriction fragment length polymorphism on PCR amplified TNFB gene products. The size marker(lane M) is DNA digested with HaeIII. Large(735bp) and small(539/196bp) fragments indicate ncoI site negative(TNFB*2 allele)and positive(TNFB*I allele), respectively.

Table 1. TNFB NcoI Polymorphism in Patients with SLE(nephritis and non-nephritis) and Normal Controls

TNFB allele	SLE (n=58) No.(%)	nephritis (n=24) No.(%)	non-nephritis (n=34) No.(%)	controls (n=141) No.(%)
Genotype frequency				
TNFB*1/TNFB*1	5(8.6)	1(4.2)	4(11.8)	25(17.7)
TNFB*1/TNFB*2	24(41.4)	10(41.7)	14(41.2)	74(52.5)
TNFB*2/TNFB*2	29(50.0)[1]	13(54.2)[2]	16(47.1)	42(29.8)
Gene frequency				
TNFB*1	34(29.3)	12(25.0)	22(32.4)	124(44.0)
TNFB*2	82(70.7)	36(75.0)	46(67.7)	158(56.0)

1: RR=2.36, p=0.011, compared to controls. 2: RR=2.78, p=0.035, compared to controls

This method was susscessfully tested on 20 homozygous B lymphoblastoid cell lines reported at the Tenth International Histoco-mpatibility Workshop (data not shown).

HLA-DRBI genotyping. HLA-DR typing of each genomic DNA was performed by polymerase chain reaction with sequence specific primers (PCR-SSP), essentially by the method of Olerup and Zetterquist with minor modifications[15]. The primers were specifically synthesized by a DNA synthesizer(Cyclone-plus, Milligen Co.) to amplify the DR alleles, according to the published nucleotide sequences of HLA class II alleles. The localizations and sequences of primers and PCR condition were the same as described in a previous report[16]. According to the fixed conditions for each DR specificity, genomic DNA were amplified using sequence specfic primers and all subjects were typed on the basis of presence or absence of the amplified product on the agarose gel. Panels of genomic DNA and standard cell lines, which were defined at the 11th International Histocompatibility Workshop(IHW) by serological method and by DNA typing using specific probes(SSO), were used to test the specificities of the primers. The results of HLA-DR typing of panel cells or cell lines showed complete concordance with results of previous serological typing and SSO typing of the IIth IHW. The generic types of DRBI alleles including 01, 15, 16, 03, 04, 11, 12, 13, 14, 07, 08, 09, 10 were determined in the study.

Statistical Analysis. Gene frequencies of the polymorphic fragments were determined by direct gene counting. Two by two tables were analyzed by X^2 or Fisher's exact test, where appropriate, with allowance for the number of comparisions made for TNFB polymorphism. Relative risks were estimated as described by Svejgaard et al[17].

RESULTS

TNFB Gene Polymorphism by NcoI PCR-RFLP

There was a singificant difference of the genotypic frequencies of TNFB alleles between patients with SLE and healthy controls(Table 1). Among three genotypes(TNFB*1 homozygote, TNFB*2 homozygote and TNFB*1/2 heterozygote), the frequency of TNFB*2 homozygote was significantly increased in patients compared with controls (50% vs 29.8%, RR=2.36, p=0.011). Although the frequency of TNFB*1 homozygote or TNFB*1/2 heterozygote was slightly decreased in patients, the difference was not statistically significant. When categorized by the presence of nephrtis and non-nephritis groups, there was no signficant difference in the distribution of TNFB alleles between nephritis and non-nephritis groups However the TNFB*2 homozygote was more significantly increased in the nephritis group(RR=2.78, p=0.035) than that in the non-nephritis patients(Table 1).

Analysis of HLA DRBI Gene

DRBI*15, a subtype of DR2, was significantly increased in patients with SLE over controls(RR=2.27, p=0.029, Table 2). DR2 was present in 36.2% of the 58 patients, compared with 20.6% of the 141 controls(RR=2.13, p=0.039). These data are quite similar to our previous report[8]. The frequencies of DRBI* 12 and * 13 were significantly decreased in patients than those in controls(RR=0.17, p=0.046; RR=0.18, p=0.001 respectively). Because the frequencies of DR2 were evenly increased in both nephritis and non-nephritis groups compared with those of controls (37.5%, 35.3% vs 18.4%, respectively), there was no statistically significant difference

Table 2. Frequencies of HLA-DRB1 Alleles in Patients with SLE(Nephritis and Non-nephritis)and Normal Controls

DRB1 Alleles	SLE (n=58) No.(%)	nephritis (n=24) No.(%)	non-nephritis (n=34) No.(%)	controls (n=141) No.(%)
DRB1*01	5(8.5)	1(4.2)	4(11.8)	13(9.2)
DR2	21(36.2)[1]	9(37.5)	12(35.3)	29(20.6)
DRB1*15	20(34.5)[2]	8(33.3)	12(35.3)	26(18.4)
DRB1*16	1(1.7)	1(4.2)	0	3(2.1)
DRB1*03	4(6.8)	1(4.2)	3(8.8)	3(2.1)
DRB1*04	13(22.4)	6(25.0)	7(20.6)	43(30.5)
DR5	8(13.8)	3(12.5)	4(11.8)	30(21.4)
DRB1*11	7(12.0)	2(8.3)	4(11.8)	17(12.1)
DRB1*12	1(1.7)[3]	1(4.2)	0	13(9.2)
DR6	14(24.1)	6(25.0)	8(23.5)	47(33.3)
DRB1*13	2(5.1)	1(4.2)	2(5.9)	33(23.4)
DRB1*14	11(18.9)	5(20.8)	6(17.7)	14(9.9)
DRB1*07	11(18.9)	4(16.7)	6(17.7)	16(11.4)
DRB1*08	18(31.0)	6(25.0)	12(35.3)	31(22.0)
DRB1*09	14(24.1)	7(29.2)	7(20.6)	32(22.7)
DRB1*10	1(1.7)	0	1(2.9)	6(4.3)

1: RR=2.13, p=0.039, compared to controls. 2: RR=2.27, p=0.029, compared to controls.
3: RR=0.17, p=0.046, compared to controls 4: RR=0.18, p=0.001, compared to controls.

Table 3. The Distribution of TNFB Genotypes after Stratification for HLA-DR2 in Patients(SLE) and Normal Controls(NC)

HLA-DR2		TNFB genotype 1/1	1/2	2/2
+	SLE	1	11	9
+	NC	5	13	8
—	SLE	4	13	20[1]
—	NC	20	61	34

1: RR=2.80, p=0.012, TNFB*2 homozygote patients vs TNFB*2 homozygote controls in DRB1 *15negatives.

Table 4. The Distribution of HLA-DR2 after Stratification for TNFB Phenotypes in Patients(SLE) and Normal Controls(NC)

TNFB genotype		DR2 +	DR2 —	RR	P
TNFB*1/1	SLE	1	4	NS	
	NC	5	20		
TNFB*1/2	SLE	11	13	3.97	0.012
	NC	13	61		
TNFB*2/2	SLE	9	20	NS	
	NC	8	34		
TNFB*1	SLE	12	17	3.18	0.019
	NC	18	81		
TNFB*2	SLE	20	33	2.74	0.010
	NC	21	95		

between nephritis and non-nephrities patients (Table 2). These results suggest that TNFB gene is a more important role for the development of nephritis in SLE than HLA DRBI gene.

Independent Analysis of the Association of TNFB Gene and DR2 with SLE

We investigated the interaction between the TNFB and the HLA DRBI alleles in patients with SLE. The interaction between TNFB and DR2 was analyzed after the stratification of TNFB and DR2(Table 3 and Table 4). TNFB*2 homozygote showed a significantly increased frequency only in DR2 negative SLE patients compared with DR2 negative controls (RR=2.80, p=0.012). Although DR2 was significantly increased in patients possessing TNFB* 1/2 heterozygote compared with controls (RR=3.97, p=0.012), the significance of DR2 in SLE was not affected by the presence of TNFB*1 or TNFB*2 allele.

DISCUSSION

Evidence for participation of genetic factors in the pathogenesis of SLE has been accumulated by many investigators. These include family aggregation of the disease, increased concordance of SLE among monozygotic and dizygoic twins, decreased red cell CRI receptors, the abnormal T cell suppressor function in healthy relatives of

SLE patients and association with several MHC loci[18-22]. Population studies have shown an increased association between SLE and HLA-DR2 and/or DR3 antigen, as well as an association with C2 and C4A deficiency of MHC class III[23,24]. The strengths of these associations vary from study to study, and they can vary in ethnically different populations[6,24,25]. In Koreans, HLA DRB1 * 1501(RR=3.3), as a subtype of DR2, and C4AQ0 allele(RR=2.1) without C4 gene deletion were reported as an increased allele in SLE patients, which was different from Causasian SLE whose predisposing MHC genes were known to be HLA DR2, DR3 and C4A gene deletion[8]. Despite these associations, the role of these molecules in SLE is not clear. Furthermore, the relative risk of SLE in a person positive for any one of these alleles has been reported to be weaker than that of some rheumatic diseases such as rheumatoid arthritis for HLA-DR4 and ankylosing spondylitis for HLA-B27[26]. Therefore, it is even questionable whether the MHC genes are the predisposing genes to the disease rather than markers for other closely linked gene(s).

Rapid progress has been made in the elucidation of the structure and function of class I and II MHC molecules, and several genes located within the MHC have been identified which are potentially involved in immunlolgic process. Because of the MHC localization of the TNFA(α) and TNFB (β) genes and the biologic activities of the gene products, it has been suggested that TNF gene loci might contribute to the susceptibility of HLA-associated autoimmune diseases[3,5]. In humans, the EcoRI RFLP of the TNFB gene is less informative than that of NcoI because of the low frequency(6%) of the less common fragments(2.5kb)[27]. In NcoI-digested genomic DNA, the TNF-β probe detects both a 5.5kb and a 5 kb, or the 10.5 kb frangment[16,28]. Despite only a small number of patients, several groups have studied the association of NcoI TNFB gene RFLP with some autoimmune diseases such as primary biliary cirrgosis(PBC), insulin deqendent diabetes mellitus(IDDM), rheumatoid arthritis(RA), primary Sjogren's syndrome(pSS) and SLE[12,13,16,29]. Among 20 patients with SLE, TNFB*1 homozygote was increased significantly when compared with the control in Fugger's[13] report. There was no difference in the phenotypic frequency for each TNFB allele in SLE, but the extremely low number of TNFB*1 homozygote in control group(6.1% vs 30%)seems to be responsible for their results. Recently, TNF NcoI-RFLP anlaysis in a larger group of SLE patients has been reported by some investigators[30,31]. Bettinotti et al[30] investigated the TNFB gene NcoI RFLP in 173 patients with German SLE and 192 unrelated controls. In that study, the phenotypic frequency of the TNFB*1 allele was significantly increased in patients compared to controls (63.6% vs 47%, RR=1.96, P<0.002). Goldstein[31] also studied the TNF NcoI-RFLP in Canadian Caucasians with SLE. Their results demonstrated that there were no differences in the frequencies of NcoI-RFLP phenotypes and genotypes between SLE and controls(TNFB*2 homozygote 38 % vs 46%, TNFB*1/2 42% vs 43%, TNFB*1 homozygote 20% vs 11% in SLE and controls, respectively).

In our study, TNFB gene analysis using NcoI RFLP showed that TNFB*2 homozygote was significantly increased in SLE compared with the controls(RR=2.36, p=0.011). These results are contradictary to other reported series. But interestingly, no significant difference was observed in the distribution of TNFB alleles between Koreans and Caucasian SLE patients even though this different distribution of TNFB alleles between Korean and Causcasian controls. Because of relatively low frequency of TNFB*2 homozygote in Korean controls compared with Caucasians, that of TNFB*2 homozygote in SLE patients seems to be increased over controls in the Korean population.

Despites the efforts of many investigators, little is known about the function of TNFB gene RFLP. Messer and coworkers[11] have reported that there is not only a structural but also a functional difference between both TNFB alleles. Upon stimulation with phytohemagglutinin(PHA) of peripheral blood mononuclear cells in vitro, TNFB*1 homozygotes are higher responders than TNFB*2 homozygotes. In mouse model systems, autoimmunity has been attributed to a low TNF-α response in NOD-mice with diabetes and mice prone for lupus nephritis(NZW x NZB)[32,33]. In humans, Jacob and coworkers reported that DR2, DQwl positive SLE patients showed low levels of TNF-α inducibilityk which was associated with an increased incidence of lupus nephritis. On the other hand, DR3-positive SLE patients are not predisposed to nephritis, and these patients have high TNF-α production. TNF mediates a variety of functions and exerts remarkably diverse effects on the immune system, which includes

the regulation of MHC class II molecule dependent on cell differentiation, and the promotion of self tolerance in early induction phase of autoimmune process rather than the late effector phase of the disease[32]. Therefore, data from experimental studies, together with the important role of TNF in immune response, could hypothesize that some clinical subset of SLE, such as lupus nephritis, might be attributed to low production of TNF which may be the effect of TNF gene polymorphism.

HLA-DR2 was a major HLA phenotype associated with SLE, which supported the previous report for Korean SLE[8]. We did not find any significant associations between TNFB alleles and HLA-DRB1*15, which were significant alleles for SLE in this study. Also there was no significant haplotypic association between TNFB alleles and C4AQ0,previously reported as an allele associated with SLE in Koreans[8](data not shown). Our data demonstrated that TNFB*2 allele was more meaningful in DR2 negative patients and DRBI*15 affected more significantly in TNFB*1/2 heterozygote. These findings suggested that TNFB*2 homozygote appeared to be an additional risk factor for SLE, espectially in DR2 negative individuals.

SLE is a heterogeneous autoimmune disorder that involves the skin, joints, serosal surface, kidney, central nervous system and blood elements. Among these various manifestations, nephritis may be the dominant clinical feature and a factor for mortality.

In this study, we categorized the patients into nephritis and non-nephritis groups to evaluate still showed a significantly increased allele over controls, whereas the significance of TNFB*2 homozygosity conferring risk for SLE was more evident in nephritis group rather than non-nephritis group. The analysis of relative risk for disease susceptibility in nephritis and non-nephritis group revealed that the relative risk of TNFB*2 homozygote in DR2 negative subjects was more prominently increased in nephritis group than in non-nephritis as a whole(RR=3.06 vs 2.65, data not shown). These observations may have implication that HLA-DR2 may play a role in SLE without nephritis rather than SLE with nephritis.

The disease entity of SLE might be heterogeneous, and the immunogenetic background might also differ among various clinical characteristics. Further study of the larger numbers of patients with different clinical features will be needed to provide more conclusive evidence on the role of TNFB gene in SLE. In addition to TNFB genotyping, we are also testing the association between TNFB genotype and production-rate phenotype in vitro. The results of these studies should also help to elucidate the significance of TNF in the pathogenesis of SLE.

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