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The Tumor Necrosis Factor β *1 Allele is Linked Significantly to HLA-DR8 in Koreans with Atrophic Autoimmune Thyroiditis who are Positive for Thyrotropin Receptor Blocking Antibody

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The localization and functional characteristics of tumor necrosis factor(TNF) β gene raise the possibility that it may be involved in the susceptibility to autoimmune thyroid diseases. To investigate whether a TNF β gene polymorphism is associated with autoimmune thyroiditis, we analyzed the TNF β gene polymorphism with the restriction enzyme NcoI in 48 Korean patients with atrophic autoimmune thyroiditis [23 were found to be thyrotropin binding inhibitor immunoglobulin(TBII) positive, 25 TBII negative], 52 goitrous autoimmune thyroiditis, and 129 healthy controls. Two TNF β alleles were identified from the restriction fragment length polymorphism studies of amplified genomic DNA.

*In atrophic autoimmune thyroiditis patients positive for TBII, 7 of 23 patients were homozygous for the TNF β *1 allele, 3 were homozygous for the TNF β *2 allele, and 13 were TNF β *1/2 heterozygous compared to controls($P=0.20$). Also, there were no associations between the TNF β gene polymorphism and either TBII-negative atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis. Of the HLA-class II antigens, the frequency of HLA-DR8 was significantly greater among the 23 Korean patients with TBII-positive atrophic autoimmune thyroiditis compared to control subjects ($Pc=0.003$). When the HLA-DR8 positive patients with TBII-positive atrophic autoimmune thyroiditis and controls were analyzed separately, the DR8 positive patients with TBII-positive atrophic autoimmune thyroiditis had more homozygotes for the TNF β *1 allele(6/12, 50.0%) and no homozygotes for the TNF β *2 allele, as compared to the DR8 negative patients with TBII-positive atrophic autoimmune thyroiditis and DR8 positive controls($P<0.05$).*

*The TNF β *1 allele linked significantly to HLA-DR8 for the development of thyrotropin receptor blocking antibody in Korean atrophic autoimmune thyroiditis patients.*

Key Words : *Tumor necrosis factor β, Atrophic autoimmune thyroiditis, HLA, thyrotropin receptor blocking antibody.*

INTRODUCTION

Tumor necrosis factor α(TNF α) is produced mainly by the macrophages and monocytes. Tumor necrosis factor β(TNF β) is a 25kDa protein produced by activated lymphocytes. These two cytokines use the same cell surface receptors and have numerous similar immunoregulatory effects (Ruddle, 1992 ; Beutler and Cerami, 1989). TNF β carries out most of the same activities as the structurally and genetically related molecule, TNF α. TNF β exerts a wide variety of effects in tissue culture ranging from killing tumor cells, to inducing gene expression, and to stimulating fibroblast proliferation (Ruddle, 1992). An association between TNF β and autoimmunity is suggested by two characteristics of the TNF β gene. First, the TNF β gene is tandemly arranged and maps between the complement genes of major histocompatibility complex(MHC) class III and class I genes on the short arm of chromosome 6(Spies et al., 1986 ; Ragoussis et al., 1988). This chromosomal location suggests the possibility that the TNF β gene might be involved in the susceptibility to autoimmune disease. Second, it has been documented that TNF, in conjunction with γ-interferon, induces MHC class II expression and enhances class I expression on thyroid cells. Thus, TNF β might be also involved functionally in the development of autoimmune thyroid disease (Buscema et al., 1989). It is known that a polymorphic NcoI restriction site is located in the first intron of the TNF β gene, with two alleles differing by one amino acid at position 26. The two TNF β alleles were defined as the TNF β *1 allele containing the NcoI restriction site (CCATGG) and AAC(Asn) at position 26 and the TNF β *2 allele lacking the NcoI restriction site and coding for ACC(Thr) (Messer et al., 1991). Recently, Badenhoop et al.(1992) demonstrated an association between the TNF β gene polymorphism and the TSH receptor antibody or HLA-DR3 positive Graves' disease in caucasians. Their work suggested that the TNF β gene polymorphism might represent an additional susceptibility marker in Graves' disease.

To investigate whether the TNF β gene polymorphism is associated with autoimmune thyroiditis, we have analyzed the TNF β gene polymorphism in Korean patients with autoimmune thyroiditis.

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MATERIALS AND METHODS

Subjects

We studied 48 consecutive Korean patients with atrophic autoimmune thyroiditis, 52 patients with goitrous autoimmune thyroiditis, and 129 healthy controls. Of the atrophic autoimmune thyroiditis patients, 23 were found to produce thyrotropin binding inhibitor immunoglobulin(TBII) and 25 patients were TBII negative. The diagnosis of autoimmune thyroiditis was based on the patient's history, physical examination, high serum TSH levels above 30 mU/L, and the presence of circulating thyroglobulin and/or microsomal autoantibodies. The diagnosis of goitrous autoimmune thyroiditis was based on the presence of palpable goiter, whereas that of atrophic autoimmune thyroiditis was based on the absence of goiter and markedly decreased radioiodine(^{131}I) thyroidal uptake(less than 4% at 24 hours). One hundred and twenty-nine healthy unrelated Koreans were randomly selected as controls. Careful analysis of their medical histories excluded autoimmune thyroid disease.

Laboratory tests

Serum T_4, T_3, and TSH concentrations were measured by RIA using commercial kits(Abbott, North Chicago, IL). The normal ranges were 85-178 nmol/L for T_4, 1.4-3.1nmol/L for T_3, and 0.38-4.1mU/L for TSH. Serum antithyroglobulin and antimicrosomal antibodies were measured in duplicate using commercial kits (R.S.R., Cardiff, United Kingdom). A titer greater than 0.3 U/mL was considered to be positive. TBII was measured in serum by radioreceptor assay using the TSH receptor antibody kit prepared by R.S.R. Ltd. (Cardiff, United Kingdom), as previously described(Southgate et al., 1984). All samples were run in duplicate. TBII activity was expressed as the percent inhibition of [^{125}I] bovine (b) TSH biding to the TSH receptor. A TBII value exceeding 15%, which was greater than 2_{SD} above the mean value from 64 normal samples, was considered to be positive. The intraassay variance in

TBII activity was 1.7-8.0% and the interassay variance was 3.7-10.5%.

HLA serotyping

HLA-A, -B, -C, and -DR typing were performed with the standard microlymphocytotoxicity technique(Terasaki and Park, 1980). The sera used were well defined and distributed by the 11th international histocompatibility workshop conference(Tsuji et al., 1992). The following specificities were included in the study : HLA-A locus, 1, 2, 3, 11, 24, 26, 30, 31, and 33 ; HLA-B locus, 7, 8, 13, 14, 27, 35, 37, 38, 39, 42, 44, 46, 47, 48, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 67, 75, and 78 ; HLA-C locus w1, w2, w3, w4, w5, w6, and w7 ; and HLA-DR locus, 1, 2, 3, 4, 5, 6, 7, 8, and 9.

TNF β gene restriction fragment length polymorphism

Genomic DNA was extracted from peripheral blood lymphocytes using the 11th international histocompatibility workshop reference protocol (Kimura and Sasazuki, 1992). Amplification of the 5' sequence of the TNF β gene was performed by polymerase chain reaction in 30 cycles ; denaturation at 94 C for an initial 6 min and then 1 min, annealing at 55 C for 30 sec, and extension at 72 C for 2 min with primers (5' primer, CCG TGC TTC GTG CTT TGG ACT A ; 3' primer, AGA GCT GGT GGG GAC ATG TCT G) in a thermocycler (Perkin-Elmer Cetus, United Kingdom) (Messer et al., 1991 ; Saiki et al., 1988). Ten microliters of amplified DNA were digested directly with 1 unit of NcoI restriction enzyme (Boehringer Mannheim GmbH, Mannheim, Germany) at 37 C for 1 hour. Electrophoresis was performed on a 1.5% agarose gel to identify the 735 base pair(bp), 539 bp, and 196 bp fragments. The amplified 735 bp fragment of genomic DNA from exon 1 to intron 3 contained the polymorphic NcoI restriction site and the variant triplet coding for amino acid 26. We named the 539 bp NcoI fragment of the TNF β gene the TNF β *1 allele and the 735 bp fragment lacking the NcoI restriction site the TNF β *2 allele.

Statistics

The frequencies of each of the HLA antigens in the patients were compared with those of the healthy unrelated control subjects using the X^2 test with Yates' correction (two-tailed) for 2×2 contingency tables. Corrected *P* values(*Pc*) were obtained by multiplying the *P* value by the number of alleles tested for each locus. Their relative risk (RR) and etiologic fraction(EF) were each calculated as de-

Table 1. HLA-DR and Cw1 antigen frequencies in autoimmune thyroiditis

HLA antigens	Controls	Atrophic autoimmune thyroiditis		Goitrous autoimmune thyroiditis
		TBII(+)	TBII(−)	
	n=136 (Cw1 : n=260)	n=23	n=24	n=62
DR1	19(14.0%)	2(8.7%)	2(8.3%)	7(11.3%)
DR2	32(23.5%)	6(26.1%)	5(20.8%)	9(14.5%)
DR3	7(5.1%)	2(8.7%)	0(0%)	1(1.6%)
DR4	50(36.8%)	9(39.1%)	14(58.3%)	28(45.2%)
DR5	23(16.9%)	1(4.3%)	0(0%)	3(4.8%)
DR6	43(31.6%)	0(0%)[a]	5(20.8%)	0(0%)[b]
DR7	19(14.0%)	1(4.3%)	4(16.7%)	8(12.9%)
DR8	22(16.2%)	12(52.2%)[c]	5(20.8%)	17(27.4%)
DR9	27(19.9%)	7(30.4%)	3(12.5%)	18(29.0%)
Cw1	60(23.5%)	14(60.9%)[d]	12(50.0%)	30(48.4)[e]

a : X^2=8.4, Pc=0.03 compared to controls.
b : X^2=23.2, Pc<0.001 compared to controls.
c : X^2=13.1, Pc=0.003 ; RR=5.7, EF=0.43 compared to controls.
d : X^2=13.7, Pc=0.002 ; RR=5.2, EF=0.49 compared to controls.
e : X^2=14.7, Pc=0.001 ; RR=3.1, EF=0.33 compared to controls.

scribed elsewhere (Svejgaard et al., 1983). *P* values of less than 0.05 were considered to be significant. Any differences in the TNF β polymorphic fragments gene frequencies between patients and controls were analyzed using the X^2 (two-tailed) or Fisher's exact test for 3×2 contingency tables.

RESULTS

HLA associations

Among the HLA class II antigens, the frequency of the DR8 antigen was significantly increased in the 23 patients with TBII-positive atrophic autoimmune thyroiditis as compared to the 136 controls (52.2% vs. 16.2% ; X^2=13.1 ; *Pc*=0.003). The relative risk was 5.7 and etiologic fraction was 0.43. Interestingly, a significantly decreased antigen frequency of HLA-DR6 was observed in patients with either TBII-positive atrophic autoimmune thyroiditis(0% vs. 31.6% ; X^2=8.4 ; *Pc*=0.03) or goitrous autoimmune thyroiditis(0% vs. 31.6% ; X^2=23.2 ; *Pc*<0.001 ; Table 1).

Among the HLA class I antigens, the frequency of the Cw1 antigen was increased significantly in patients with either TBII-positive atrophic autoimmune thyroiditis(60.9% vs. 23.5% ; X^2=13.7 ; *Pc*=0.002 ; RR=5.2 ; EF=0.49) or goitrous autoimmune thyroiditis (48.4% vs. 23.5% ; X^2=14.7 ; *Pc*=0.001 ; RR=3.1 ; EF=0.33), as compared to controls(Table 1). However, the HLA-A and -B antigens were not associated significantly with autoimmune thyroiditis.

TNF β gene polymorphism

Seven (30.4%) out of 23 patients with TBII-positive atrophic autoimmune thyroiditis were homozygous for the TNF β*1 allele, whereas 13 (56.5%) were heterozygous, and 3 (13.0%) were homozygous for the TNF β*2 allele. In TBII-negative atrophic autoimmune thyroiditis, 6 (24.0%) out of 25 patients were TNF β*1 allele homozygotes, 12 (48.0%) heterozygotes, and 7(28.0%) TNF β*2 allele homozygotes. Eleven (21.2%) out of 52 patients with goitrous autoimmune thyroiditis were homozygous for the TNF β*1 allele, 20 (38.5%) heterozygous, and 21 (40.4%) homozygous for the TNF β*2 allele. By comparison, 28(21.7%) of the 129 control subjects were homozygous for the TNF β*1 allele, 61(47.3%) heterozygous, and 40(31.0%) homozygous for the TNF β*2 allele(Table 2). The patients with TBII-positive atrophic autoimmune thyroiditis had more homozygotes for the TNF β*1 allele and heterozygotes for the TNF β*1/2 allele and fewer homozygotes for the TNF β*2 allele than the control subjects. However, these associations were not statistically significant (*P*=0.20). Also, there were no significant associations between the TNF β gene polymorphism and either TBII-negative atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis.

Among HLA-class II antigens, the frequency of the DR8 antigen was increased significantly in 23 Korean patients with TBII-positive atrophic autoimmune thyroiditis, as compared to control subjects (Table 1). Therefore, in order to test whether the TNF β gene polymorphism is related to HLA-DR8, we analyzed the presence of this polymorphism in DR8 positive and negative patients with TBII-positive atrophic autoimmune thyroiditis. Of the 12 DR8 positive patients with TBII-positive atrophic autoimmune thyroiditis, six(50.0%) were homozygous for the TNF β*1 allele, 6(50.0%) were heterozygous for the

Table 2. Distribution of the TNF β gene polymorphism in patients with autoimmune thyroiditis

	TNF β*1 homozygous	TNF β*1/2 heterozygous	TNF β*2 homozygous	Total
Atrophic autoimmune thyroiditis				
TBII(+)	7(30.4%)	13(56.5%)	3(13.0%)	23
TBII(−)	6(24.0%)	12(48.0%)	7(28.0%)	25
Goitrous autoimmune thyroiditis	11(21.2%)	20(38.5%)	21(40.4%)	52
Controls	28(21.7%)	61(47.3%)	40(31.0%)	129

TBII : Thyrotropin binding inhibitor immunoglobulin

Table 3. TNF β gene polymorphism in DR8 positive patients with TBII positive atrophic autoimmune thyroiditis and controls

	TNF β∗1 homozygous	TNF β∗1/2 heterozygous	TNF β∗2 homozygous	Total
TBII(+) Atrophic autoimmune thyroiditis				
DR8(+)[a,b]	6(50.0%)	6(50.0%)	0(0%)	12
DR8(−)	1(9.1%)	7(63.6%)	3(27.3%)	11
Controls				
DR8(+)	4(19.0%)	10(47.6%)	7(33.3%)	21
DR8(−)	23(21.3%)	52(48.1%)	33(30.6%)	108

TBII : Thyrotropin binding inhibitor immunoglobulin
a : DR8(+) patients vs DR8(+) controls ; X^2=6.4, P=0.04
b : DR8(+) patients vs DR8(−) patients ; X^2=6.6, P=0.036

TNF β∗1/2 allele, and none were homozygous for the TNF β∗2 allele, as compared to both the DR8 negative patients with TBII-positive atrophic autoimmune thyroiditis and DR8 positive controls(X^2=6.6 ; *P*=0.036 in DR8 negative patients with TBII-positive atrophic autoimmune thyroiditis ; X^2=6.4 ; *P*=0.04 in DR8 positive controls ; Table 3). However, the TNF β gene polymorphism was not associated with HLA-Cw1 in autoimmune thyroiditis (data not shown). These results identify a significant linkage between the TNF β∗1 allele and HLA-DR8 in Korean patients with TBII-positive atrophic autoimmune thyroiditis.

DISCUSSION

The TNF β gene resides on chromosome 6 between the complement genes of the MHC class III and class I genes (Spies et al., 1986 ; Ragoussis et al., 1988). In conjunction with γ-interferon, TNF induces MHC class II and enhances class I expression on the thyrocytes (Buscema et al., 1989). The location and functional characteristics of the TNF β gene raises the possibility that this gene may be involved in the susceptibility to autoimmune thyroid disease. Previously, we reported that the prevalence of the blocking type antibody to the TSH receptor (TRBab) in atrophic autoimmune thyroiditis is 55% by TBII assay and 75% by thyroid stimulation blocking antibody (TSBab) assay (Cho et al., 1989), this association is higher than those of the previous reports (Tamaki et al., 1990). We have reported that the antigen or allelic frequencies of HLA-DR8 and HLA-DQB1*0302 were increased significantly in TRBab-positive atrophic autoimmune thyroiditis compared to controls (Cho et al., 1993). These findings suggested that atrophic autoimmune thyroiditis is immunogenetically heterogeneous according to the presence or absence of TRBab and TRBab may have a major role in the development of hypothyroidism and thyroid atrophy, particularly in those patients with TRBab-positive atrophic autoimmune thyroiditis (Cho et al., 1989 ; Konishi et al., 1983). We have, therefore, analyzed the TNF β gene polymorphism in Korean patients with TRBab-positive atrophic autoimmune thyroiditis to determine whether the TNF β gene polymorphism is associated with TRBab-positive atrophic autoimmune thyroiditis. We found no association between the TNF β gene polymorphism and TRBab-positive atrophic autoimmune thyroiditis. However, when HLA-DR8 positive patients with TRBab-positive atrophic autoimmune thyroiditis were analyzed separately, HLA-DR8 and TRBab-positive atrophic autoimmune thyroiditis had a strong association to the TNF β∗1 allele compared to DR8 positive controls and DR8 negative patients with TRBab-positive atrophic autoimmune thyroiditis. This result suggests that the TNF β gene, acting in conjunction with MHC class II genes in patients with TRBab-positive atrophic autoimmune thyroiditis, may contribute to the development of atrophic autoimmune thyroiditis. And this result futher supports the possibility that TRBab-positive atrophic autoimmune thyroiditis is immunogenetically different from both goitrous autoimmune thyroiditis and TRBab-negative atrophic autoimmune thyroiditis (Cho et al., 1993).

Badenhoop et al. (1992) have reported that patients with TSH receptor antibodies positive Graves' disease had more heterzygotes for the TNF β∗1/

2 allele and less homozygotes for the TNF β*2 allele than controls. When they analyzed HLA-DR3 positive patients and controls separately, TNF β*1/2 heterozygotes were still significantly increased in the patients with DR3 positive Graves' disease as compared to DR3 negative patients with Graves' disease and controls. They suggested that Graves' patients heterozygous for the TNF β*1/2 allele might represent an immunologic subset of the disease, the TNF β*1/2 allele may represent an additional susceptibility marker in Graves' disease in caucasians. We reported previously that the antigenic frequency of HLA-DR8 was also increased significantly in Korean patients with Graves' disease compared to controls (29.7% vs 15.5% ; *Pc*=0,02 ; RR=2.3) (Cho et al., 1987). This finding seems to suggest that there may be disease susceptibility genes common to Graves' disease and TRBab-positive atrophic autoimmune thyroiditis. Therefore, we can speculate that the TNF β gene polymorphism may be associated with TSH receptor-related autoimmune thyroid disease in conjunction with the MHC class II genes.

Interestingly, we found that the antigenic frequency of HLA-Cw1 was significantly increased in all patients with autoimmune thyroiditis regardless of the presence or absence of TRBab. Because cytotoxic T cells are restricted by MHC class I, we speculated that HLA-Cw1 may be involved in the immunologic destruction of thyrocytes in Koreans. Although we did not perform a functional assay on TNF β in this study, Messer et al.(1991) reported that the TNF β*1 allele is strongly associated with an increase in the TNF β production by lymphocytes in response to phytohemagglutinin. When HLA-Cw1 positive patients with autoimmune thyroiditis were analyzed, there was no association between the TNF β gene polymorphism and HLA-Cw1 positive autoimmune thyroiditis. Therefore, we speculate that HLA-Cw1 may be a genetic marker for autoimmune thyroiditis rather than encode a functional effect.

In summary, we found that the antigenic frequency of HLA-DR8 was increased significantly in patients with TBII-positive atrophic autoimmune thyroiditis and the TNF β*1 allele was significantly linked to HLA-DR8 in patients with TBII-positive atrophic autoimmune thyroiditis. It is possible that the TNF β*1 allele, in conjunction with HLA-DR8 antigen, may be related to susceptibility markers responsible for the production of TRBab in Korean atrophic autoimmune thyroiditis patients.

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