Fukushima J. Med. Sci.,
Vol. 52, No. 2, 2006

[**Original Article**]

POSSIBLE ASSOCIATION OF CYTOTOXIC T LYMPHOCYTE ANTIGEN-4 GENETIC POLYMORPHISM WITH LIVER DAMAGE OF PRIMARY BILIARY CIRRHOSIS IN JAPAN

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(Received June 12, 2006, accepted August 17, 2006)

Abstract: Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important inhibitor of T-lymphocyte response. Polymorphisms in the CTLA-4 gene have been reported to be associated with numerous autoimmune diseases. The aim of this study was to determine whether polymorphisms of CTLA-4 exon 1 (+49) genes are associated with susceptibility and clinicolaboratory findings of primary biliary cirrhosis (PBC) in the Japanease population. Blood samples were obtained from 45 patients (6 men and 39 women, aged 23-56 years) with PBC and 73 healthy controls (48 men and 25 women, aged 22-72 years). CTLA-4 exon 1 (+49) polymorphism was defined using a polymerase chain reaction-restriction fragment length polymorphism with Bst71I restriction enzyme. The genotype frequencies of A/A, A/G, and G/G in 45 patients with PBC were 11% (5 patients), 44% (20 patients), and 44% (20 patients), respectively. There was no significant difference between frequencies in PBC patients and healthy controls. PBC patients with G/G genotype had significantly higher serum levels of ALT, GGT, and IgM than those in patients with A/A or A/G genotype. In conclusion, CTLA-4 gene polymorphisms are not associated with susceptibility of PBC in Japan; however, G/G genotype may be associated with liver damage.

Key words: Cytotoxic T lymphocyte antigen-4, polymorphysm, primary biliary cirrhosis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an idiopathic liver disease characterized by progressive destruction of intrahepatic bile ducts and possibly caused by autoimmune reactions[1]. It is generally believed that cytotoxic T cells in the biliary epithelial layer play an important role in biliary epithelial destruction[2].

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is involved in the regulation of T cells and its antigen is only expressed on activated T cells. which binds to CD80 molecules on antigen-presenting cells[3,4]. This binding delivers negative signals to T cells that affect T cell proliferation, cytokine production, and immune responses. The CTLA-4 gene is located on chromosome 2q33 and three CTLA-4 gene polymorphisms in exon 1 (adenine or guanine at position) and in promoter -318, and a microsatellite (AT) n marker at position 642 of the 3'-untranslated region of exon 3[5,6]. CTLA-4 gene polymorphisms have been shown to be associated with type 1 diabetes[7], Graves' disease[7], celiac disease[8], and Addison's disease[9]. CTLA-4 exon 1 (+49) genes G allele and G/G genotype are reported to confer genetic susceptibility to these diseases. In addition, CTLA-4 (+49) genes are reported that GG genotype is associated with more severe cell dysfunction in type 1 diabetes[10].

In this study, we investigated whether polymorphisms of CTLA-4 exon 1 (+49) genes are associated with susceptibility and clinicolaboratory findings of PBC in the Japanese population.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 45 patients (6 men and 39 women, aged 23-56 years) with PBC and 73 healthy controls (48 men and 25 women, aged 22-72 years). PBC was diagnosed by either histology of liver biopsy specimens or clinical findings of anti-mitochondrial antibodies (AMA) and cholestatic dysfunction of the liver followed by jaundice or puritus, based on 'Criteria for diagnosis of PBC in Japan' by the Study Group for Autoimmune Hepatitis, a subdivision of the Research Group for Intractable Hepatitis sponsored by the Ministry of Health and Welfare of Japan[11]. Blood was collected in EDTA tubes and DNA from peripheral blood mononuclear cells was isolated using the Genomic DNA purification kit (Gentra systems, Minnesota, USA).

In sera from patients with PBC, AMA was simultaneously detected by indirect immunofluorescence using frozen sections of a rat kidney. All subjects gave written informed consent to participate in this study.

Polymorphism typing of CTLA-4 exon 1 (+49)

CTLA-4 exon 1 (+49) polymorphism was defined using a polymerase chain

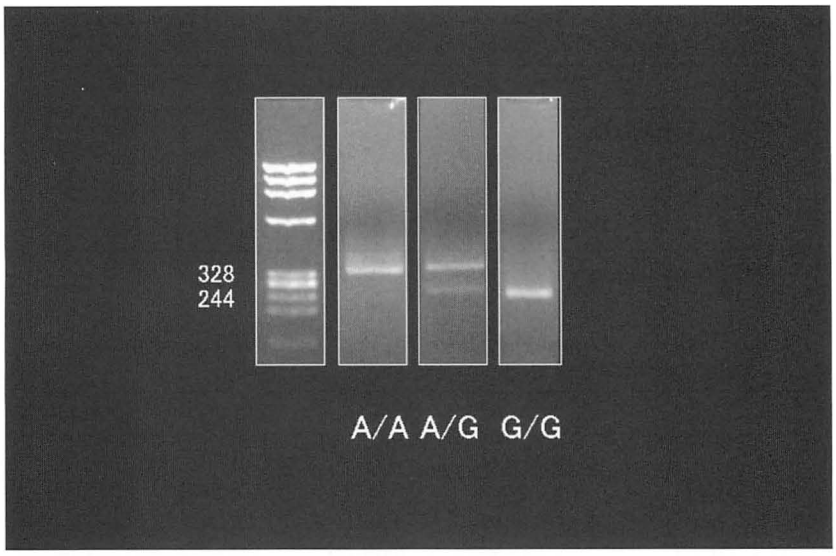

Figure. 1. Representative agarose gel electropheresis illustrating PCR-RFLP products for the CTLA-4 exon 1 (+49) polymorphism.

reaction-restriction fragment length polymorphism (PCR-RFLP) with Bst71I restriction enzyme, according to the methods reported by Agarwal *et al.*[12]. Briefly, PCR was carried out using a forward primer 5′-CCACGGCTTCCTTTCTCGTA-3′ and a reverse primer 5′-AGTCTCACTCACCTTTGCAG-3′. Using a Parkin Elmer thermal cycler, samples were subjected to initial denaturation for 2 min at 95°C, 40 cycles for 30s at 94°C for denaturing, 45s at 50°C for annealing and 30s at 72°C for extension. A 328-bp fragment containing +49 A/G polymorphism in exon 1 of CTLA-4 was amplified. The substitution created a Bst71I restriction site in G allele. Amplified products were incubated at 65°C for 2 h using 2 U of Bst71I per reaction. Digested products were electrophoresed on a 2.0% agarose gel. Digested G allele yielded fragments of 244 bp and 84 bp, and an allele yielded a 328-bp fragment (Figure 1).

Statistical analysis

Results are expressed as means±SD. Statistical analysis of the data was performed using the chi-square test, two-tailed Welch's *t*-test and Kruskal-Wallis rank test. Differences with a P value <0.05 were considered significant.

RESULTS

Genotype frequencies

The genotype frequencies of A/A, A/G, and G/G at exon 1 (+49) on CTLA-4 gene in 45 patients with PBC were 11% (5 patients), 44% (20 patients), and 44%

Table 1. Genotype frequencies at exon 1 (+49) on CTLA-4 gene in PBC patients and healthy controls

	PBC ($N=45$)	Controls ($N=73$)	P values
Genotype frequency			
A/A	5 (11%)	14 (19%)	NS
A/G	20 (44%)	33 (45%)	NS
G/G	20 (44%)	26 (36%)	NS

NS, not significantly different.

Table 2. Clinicolaboratory findings in PBC patients according to the genotype variations at exon 1+49 on CTLA-4 gene

		A/A	A/G	G/G	P values
Age		51±13	51±12	51±11	NS
Sex (M/F)		0/5	4/16	3/17	NS
T-Bil (mg/dl)		1.4±0.8	1.2±1.7	1.3±1.8	NS
ALT (IU/l)		55±38	100±148	124±284	<0.05
GGT (IU/l)		180±129	227±253	301±321	<0.05
IgG (mg/dl)		1,940±459	2,132±611	2,011±778	NS
IgM (mg/dl)		390±94	444±154	585±427	<0.05
AMA frequency		80%	72%	70%	NS
ANA frequency		80%	89%	70%	NS
Histology (Scheuer's stage)	I	2	10	10	NS
	II	2	7	1	
	III	0	1	4	
	IV	1	2	5	

NS, not significantly different. Normal ranges: T-Bil (0.4-1.2 mg/dl), ALT (6-29 IU/l), GGT (7-55 IU/l), IgG (910-1,910 mg/dl), IgM (36-200 mg/dl).
P values were compared with G/G group vs. A/A or A/G group.

(20 patients), respectively, as shown in Table 1. Those in 73 healthy controls were 19%[14], 45%[33], and 36%[26], respectively. There was no significant difference between frequencies in the two.

Genotype frequency and clinicolaboratory findings

As shown in Table 2, PBC patients with G/G genotype at exon 1 (+49) on CTLA-4 gene had significantly higher in serum levels of ALT, GGT, and IgM than those in patients with A/A or A/G genotype. However, there were no significant differences in other clinicolaboratory findings according to the three genotypic variations. In histological stage, there were no significant differences.

DISCUSSION

There have been some in which CTLA-4 gene polymorphisms in patients with PBC were assessed[12-15]. Studies conducted in the UK, USA and China have shown that CTLA-4 gene polymorphisms are associated with susceptibility of PBC[12,14,15]. However, a study carried out in Brazil showed no association[13]. In the present study, there were no significant differences between PBC patients and healthy controls in the genotype and allele frequencies at exon 1 (+49) on the CTLA-4 gene. It is also necessary to consider the difference in race. However, a study in China showed that allelic variation at the +49 site of CTLA-4 exon 1 was significantly associated with PBC and that the frequency of G alleles was increased in patients with PBC compared with that in controls[14]. In our study, G allelic variations were found in 67% of the PBC patients and 58% of the controls.

There has been no report to date of a significant association between CTLA-4 gene polymorphisms and clinicolaboratory findings in patients with PBC. In this study, we showed that PBC patients with G/G genotype at exon 1 (+49) on the CTLA-4 gene had significantly higher serum levels of ALT, GGT, and IgM than those in patients with A/A or A/G genotype. The CTLA-4 molecule is an important inhibitor of T-lymphocyte response. T cell respones would certainly participate in the pathogenesis of PBC, as judged by histochemical staining of tissue samples, and by analyzing T cell lines that proliferate in the presence of putative mitochondrial autoantigens. Kouki *et al.* reported that the inhibitory effect of CTLA-4 on T cells was less potent in cells from subjects with (+49) G/G than A/A alleles, and suggested that this particular polymorphism was the actual disease-associated allele[16]. Thus, PBC patients with G/G genotype at exon 1 (+49) may be more strongly injured by T cells. In this study, there was no significant difference in histological stage and CTLA-4 genotype. Serial clinical analysis is needed in order to evaluate whether G/G genotype at exon 1 (+49) is associated to development of PBC. Polymorphisms in the CTLA-4 gene have been reported to be associated with numerous autoimmune diseases; however, other diseases such as Graves' disease[17] and idiopathic hypoparathyroidism[18] showed no significant association in clinicolaboratory findings. Thus, examination of clinical usefulness is also needed in future.

There have been many reports of other immunorelated gene polymorphisms in patients with PBC such as polymorphisms in mannose-binding lectin[19], cytokeratin-19 pseudogene[20], interleukin-10 promotor gene[21], endothelial nitric oxide synthesis gene[22], and toll-like receptor-9 gene[23]. All of those studies showed an association with susceptibility of PBC, but analysis of single gene polymorphism induces limit. We think that analysis by genetic assembly becomes necessary instead of by genetic single analysis in order to make clear genetic association in patients with PBC.

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