Ligands for Programmed Cell Death 1 Gene in Patients with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To investigate the role of ligands for programmed cell death 1 (PD-L) in the pathogenesis of systemic lupus erythematosus (SLE).

Methods. One hundred sixty-four patients with SLE and 160 healthy controls were enrolled in our study. The *PD-L1* and *PD-L2* polymorphisms were determined by polymerase chain reaction (PCR)/direct sequencing or restriction fragment length polymorphism (RFLP)-PCR.

Results. The genotype distributions of *PD-L2* 47103 C/T polymorphisms in patients with SLE were significantly different from those of the controls (p = 0.003). The genotype frequency of *PD-L2* 47103 T/T, in comparison with 47103 C/C, was significantly increased in patients with SLE when compared with that of the controls (odds ratio 2.5, 95% confidence interval 1.4–4.4, p = 0.001). A similar finding could also be found in the allele frequency of *PD-L2* 47103 T (SLE vs control, OR 1.7, 95% CI 1.3–2.4, p = 0.001). There were no significant differences in the genotype and allele frequencies of *PD-L1* polymorphisms between the patients and controls.

Conclusion. *PD-L2* 47103 T may be associated with susceptibility to SLE in Taiwan. (First Release Mar 1 2007; J Rheumatol 2007;34:721–5)

Key Indexing Terms:
PROGRAMMED CELL DEATH 1 LIGAND
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SYSTEMIC LUPUS ERYTHEMATOSUS
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Systemic lupus erythematosus (SLE) is a prototype of autoimmune diseases, which frequently involves multiple organs. The detailed pathogenesis is still obscure. Many genes such as human leukocyte antigen, complement, FcrR, mannose binding lectin, protein tyrosine phosphatase N22, cytokines, and chemokine genes have been described to be associated with the development of SLE[1-5].

Programmed cell death 1 (PD-1) is an immunoinhibitory receptor expressed by activated T cells, B cells, and myeloid cells[6]. The ligands for PD-1 (PD-L1 and PD-L2, also known as B7-H1 and B7-DC) are type I transmembrane proteins structurally related to the B7 family. They can be induced in monocytes, dendritic cells, endothelial cells, keratinocytes, and B cells[6-10]. However, PD-L1 expression is different from that of PD-L2. PD-L1 is also expressed on activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells. In contrast, PD-L2 can also be presented on placental endothelium and medullary thymic epithelial cells[10]. The interactions of PD-1 with PD-L1 and PD-L2 result in the inhibition of T cell receptor-mediated lymphocyte proliferation and cytokine secretion, and they also inhibit CD28-mediated costimulation. The relative levels of inhibitory PD-L1 and costimulatory CD80/CD86 signals on antigen-presenting cells determine the extent of T cell activation and the threshold between tolerance and autoimmunity. Therefore, PD-L1 expression on nonlymphoid tissues and its interaction with PD-1 may determine the extent of immune responses at sites of inflammation[6].

Recent studies using anti-PD-L1 monoclonal antibodies have suggested a role for PD-L1 in regulating autoimmune diseases. Blockade of the PD-L during experimental autoimmune encephalomyelitis (EAE) or diabetes exacerbates the diseases[11,12]. PD-L1 blockade rapidly precipitated diabetes in prediabetic female non-obese diabetic (NOD) mice. PD-L2 blockade in animals also resulted in augmentation of EAE. These studies showed that PD-1–PD-L blockade was related to the development of autoimmune disease.

The polymorphisms in exons of *PD-L1* and *PD-L2* may result in amino acid substitution, structural changes, and expression of PD-L1 and PD-L2. The consequent interactions

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between PD-1 and its ligands may also be changed. Therefore, *PD-L1* and *PD-L2* polymorphisms may be related to the pathogenesis of autoimmune diseases. *PD-1* polymorphisms may be associated with susceptibility to SLE[13]. Therefore, *PD-L1* and *PD-L2* polymorphisms may also be associated with susceptibility to SLE. Our purpose was to investigate the associations of *PD-L1* and *PD-L2* polymorphisms with susceptibility and clinical manifestations of SLE in Taiwan.

MATERIALS AND METHODS

One hundred sixty-four patients with SLE (148 female, 16 male) and 160 healthy controls (139 female, 21 male) were enrolled in our study. All patients and controls were Taiwanese. The diagnosis of SLE was according to the American College of Rheumatology (ACR) 1997 revised criteria for the classification of SLE[14]. Our study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital.

There are 3 polymorphisms in the exons of *PD-L1* including *PD-L1* 1072 G/C (rs 12551333; exon 3, amino acid 49, Asp → His), 1113 G/A (rs 4278201; exon 3, amino acid 62, Lys → Lys, synonymous), and 6777 C/G (rs 17718883; exon 4, amino acid 146, Pro → Arg). The polymorphisms of *PD-L1* 1072 C/G and 1113 A/G were determined by polymerase chain reaction (PCR)/direct sequencing. The sequences of primers are shown in Table 1. PCR was performed under the following conditions: initial denaturation at 96°C for 3 min and 5 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, and then 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. A final extension phase was also performed at 72°C for 7 min. Then the nucleotide sequence was determined by the method of direct sequencing with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The polymorphisms of *PD-L1* 6777 C/G were determined by the restriction fragment length polymorphism (RFLP)-PCR method. The sequences of primers and restriction enzymes are shown in Table 1. The amplification conditions consisted of initial denaturation at 95°C for 3 min, followed by 5 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and then a final extension phase at 72°C for 7 min. The restriction enzyme Bsr I was used to determine the 6777 C/G polymorphisms.

There are 3 non-synonymous polymorphisms in *PD-L2* including *PD-L2* 24293 G/C (rs 12339171; exon 3, amino acid 58, Ser → Thr), 47103 C/T (rs 7854303; exon 5, amino acid 229, Ser → Phe), and 47139 C/T (rs 7854413; exon 5, amino acid 241, Thr → Ile). These polymorphisms were also determined by RFLP-PCR. The sequences of primers and restriction enzymes are shown in Table 1. To determine the *PD-L2* 24293 G/C polymorphisms, PCR was carried out under the following conditions: initial denaturation at 95°C for 3 min and 5 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, and then 35 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. A final extension phase was also performed at 72°C for 7 min. Then the PCR product was digested with Bsr I.

A mismatched nucleotide (underlined) was used in the downstream primer to determine 47103 C/T and 47139 C/T. The amplification conditions consisted of initial denaturation at 96° C for 3 min, followed by 5 cycles of denaturation at 95° C for 1 min, annealing at 56° C for 1 min, and extension at 72° C for 1 min, and 30 cycles of denaturation at 95° C for 1 min, annealing at 54° C for 1 min, and extension at 72° C for 1 min, and then a final extension phase at 72° C for 7 min. The restriction enzymes Bcc I and Tsp45 I were used to determine the 47103 C/T and 47139 C/T polymorphisms, respectively. The *PD-L2* 47103 C/T and 47139 C/T polymorphisms were also confirmed in some cases by direct sequencing with the BigDye Terminator Cycle Sequencing Kit. The sequences of primers were 5'- CCT GTT GGT CTA CCT CTT AG- 3' and 5'- TGA AAG CAG CAA GCC ATA GG- 3'. The amplification conditions consisted of initial denaturation at 96°C for 3 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and then a final extension phase at 72°C for 5 min.

The clinical manifestations of SLE were defined according to the 1997 revised ACR criteria. The severity of SLE was determined according to the SLICC/ACR damage index for SLE[15].

The chi-square test (with Yates correction for 2×2 table) or Fisher's exact test was used for statistical analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using the SPSS program.

RESULTS

The distributions of *PD-L1* and *PD-L2* genotypes were compatible with Hardy-Weinberg equilibrium in the controls. The *PD-L1* 1072 C and *PD-L1* 1113 A alleles could not be detected in Taiwanese. Moreover, there was no significant difference in the genotype frequency of *PD-L1* 6777 C/G polymorphisms between the patients with SLE and controls (Table 2). Similar findings could also be found regarding their allele frequencies.

PD-L2 24293 C allele could not be found in Taiwanese patients and controls in our study. All of the patients and controls were *PD-L2* 24293 G. The study revealed that the genotype distributions of *PD-L2* 47103 C/T polymorphisms in the patients with SLE were significantly different from those of

Table 1. The sequences of primers and detection methods in determining *PD-L1* and *PD-L2* polymorphisms.

Polymorphisms	Primers	Detection Methods or Restriction Enzymes
PD-L1		
1072 G/C and	5'-TGT GGT AGA GTA TGG TAG C-3'	Direct sequencing
1113 G/A	5'-CTG TCT GTA GCT ACT ATG C-3'	
6777 C/G	5'-TAC GTA GTT CTG TGC TCA G-3'	Bsr I
	5'-GTT GAT TCT CAG TGT GCT G-3'	
PD-L2		
24293 G/C	5'-AGC ATG GCA GCA ATG TGA C-3'	Bsr I
	5'-CAC TCA CCT TTG ACT TTC AG-3'	
47103 C/T	5'-GCT TCA CAT TTT CAT CCC AT-3'	Bcc I
	5'-AGT GGC TCA TGT GCA GAC-3'	
47139 C/T	5'-GCT TCA CAT TTT CAT CCC AT-3'	Tsp45 I
	5'-AGT GGC TCA TGT GCA GAC-3'	

Table 2. Frequencies of *PD-L1* polymorphisms in patients with SLE and controls. The *PD-L1* 1072 C and *PD-L1* 1113 A alleles could not be detected in Taiwanese. There were no significant differences in the genotype and allele frequencies of *PD-L1* 6777 polymorphisms between the patients with SLE and controls.

Polymorphisms of *PD-L1*	SLE, n = 164 (%)	Controls, n = 160 (%)
Genotype		
1072 G/G	164 (100)	160 (100)
1113 G/G	164 (100)	160 (100)
6777 C/C	124 (75.6)	127 (79.4)
C/G	39 (23.8)	31 (19.4)
G/G	1 (0.6)	2 (1.2)
Allele		
1072 G	328 (100)	320 (100)
1113 G	328 (100)	320 (100)
6777 C	287 (87.5)	285 (89.1)
G	41 (12.5)	35 (10.9)

the controls (p = 0.003; Table 3). The genotype frequency of *PD-L2* 47103 T/T, in comparison with 47103 C/C, was significantly higher in the patients with SLE than that of the controls (OR 2.5, 95% CI 1.4–4.4, p = 0.001). The allele frequency of *PD-L2* 47103 T was also significantly increased in patients with SLE compared with controls (OR 1.7, 95% CI 1.3–2.4, p = 0.001). However, there were no significant differences in the genotype and allele frequencies of *PD-L2* 47139 T/C polymorphisms between the patients with SLE and controls.

Our study also revealed that *PD-L1* and *PD-L2* polymorphisms were not associated with the clinical manifestations of SLE including nephritis, central nervous system involvement, cutaneous vasculitis, and various autoantibodies such as the antinuclear antibody, anti-dsDNA, anti-Sm, and anti-RNP. The *PD-L1* and *PD-L2* polymorphisms were also not associated with the SLICC/ACR damage index for SLE (data not shown).

DISCUSSION

Our study showed that *PD-L2* 47103 T was associated with susceptibility to SLE in Taiwan.

PD-L1 is constitutively expressed in T cells, B cells, macrophages, and dendritic cells, and is upregulated following activation of these cells. PD-L1 is also expressed on nonlymphoid cells including cardiac endothelial cells, pancreatic islet cells, brain glial cells, and muscle cells. PD-L2 expression is observed on activated macrophages and dendritic cells[16,17]. Moreover, PD-L1 and PD-L2 are expressed in various tumor cells, such as ovary, esophagus, kidney, and brain tumors[18-24].

The PD-1–PD-L pathway regulates the immune response in both lymphoid and nonlymphoid organs. The interactions between PD-1 and PD-L inhibit lymphocyte activation. Although positive effects of PD-1–PD-L interaction have been found[8,9,25,26], the negative effects are well documented. It is still unknown whether the positive effects are caused by the inhibition of negative signaling or by other stimulatory receptors.

Mice deficient in PD-1 developed spontaneous autoimmune diseases, which suggested a negative costimulatory function. C57 BL/6-Pdcd1$^{-/-}$ mice developed lupus-like glomerulonephritis and arthritis[27]. BALB/c-Pdcd1$^{-/-}$ mice developed dilated cardiomyopathy with production of autoantibodies against cardiac troponin I[28-30]. In 2C-Pdcd1$^{-/-}$ H-2$^{b/d}$ mice, the mice died of the graft-versus-host-like disease[27]. Therefore, the PD-1–PD-L pathway may play a role in the induction and maintenance of peripheral tolerance. PD-1–PD-L interaction inhibits adverse immune response in 2 ways. PD-L on antigen-presenting cells inhibits T cell activation and induces peripheral tolerance. Moreover, PD-L on target cells inhibits the effector function of T cells to maintain tolerance.

PD-L on nonlymphoid organs prevents tissue destruction by suppressing the effector function of autoreactive lymphocytes. PD-L1 on islet cells suppresses the effector function of diabetogenic T cells[31]. Moreover, antibody blockade of the

Table 3. Frequencies of *PD-L2* polymorphisms in patients with SLE and controls. The *PD-L2* 24293 C allele could not be found in this study.

Polymorphisms of *PD-L2*	SLE, n = 164 (%)	Controls, n = 160 (%)	p (for overall genotype frequency)	p (for individual genotype and allele frequency)	OR (95% CI)
Genotype					
24293 G/G	164 (100)	160 (100)	NS		
47103 C/C	63 (38.4)	77 (48.1)	0.003		1
C/T	46 (28.0)	56 (35.0)			
T/T	55 (33.5)	27 (16.9)		0.001	2.5 (1.4–4.4)
47139 T/T	151 (92.1)	144 (90.0)	NS		
C/T	13 (7.9)	15 (9.4)			
C/C	0 (0)	1 (0.6)			
Allele					
24293 G	328 (100)	320 (100)	NS		
47103 C	172 (52.4)	210 (65.6)			
T	156 (47.6)	110 (34.4)		0.001	1.7 (1.3–2.4)
47139 T	315 (96.0)	303 (94.7)	NS		
C	13 (4.0)	17 (5.3)			

PD-1–PD-L pathway in prediabetic NOD mice also induced type I diabetes[12]. Wang, *et al* revealed that NOD-Pdcd1$^{-/-}$ mice also developed type I diabetes[32]. It can therefore be concluded that PD-1–PD-L interaction may play a role in preventing the development of diabetes. PD-L1 on tumor cells suppresses the cytolytic activity of CD8+ T cells[33,34]. Blocking PD-1–PD-L interaction will accelerate tumor eradication. A correlation between PD-L expression on tumor cells and poor prognosis has been found for human cancer patients[20,23]. Therefore a PD-1–PD-L blockade may activate the immune system of tumor-bearing hosts to eradicate tumors.

PD-L1 on antigen-presenting cells results in inactivation of T cells. Resting dendritic cells induce inactivation or anergy of T cells and CD8+ T cell tolerance through the PD-1–PD-L pathway[35]. Virus- or parasite-infected cells will induce PD-L1 expression on dendritic cells, and then induce T cell anergy, which will result in immune paralysis against viruses or parasites[36,37]. However, activated dendritic cells express lower levels of PD-L than costimulatory and MHC molecules. The activating signals overcome the inhibitory signal of PD-1, and T cell activation will result.

PD-1–PD-L interaction inhibits adverse immune responses to prevent autoimmunity. The *PD-1* or *PD-L* polymorphisms may interfere with the interaction between PD-1 and PD-L, and then diminish the prevention of autoimmune response. Some studies revealed that *PD-1* polymorphisms were associated with several immune-mediated diseases including rheumatoid arthritis, SLE, and type I diabetes[13,38-41]. Therefore *PD-L* polymorphisms may also be related to the development of autoimmune diseases. A report in regard to the association of *PD-L* polymorphisms with SLE is still unavailable. Our study showed that the polymorphisms in the exons of *PD-L1* were not associated with susceptibility to SLE. However, the *PD-L2* 47103 T was related to the development of SLE. *PD-L2* 47103 C/T are non-synonymous polymorphisms. *PD-L2* 47103 C encodes an amino acid serine in the transmembrane region. However, *PD-L2* 47103 T encodes amino acid phenylalanine, which contains an aromatic ring. The expression of protein molecule on the cell surface may be regulated by its transmembrane domain[42]. Transmembrane domain also plays a role in dimerization or oligomerization for surface molecules[43,44]. Similar to a mutation in CD80, the amino acid substitution of PD-L2 may result in PD-L2 molecule expression on cell surface in small clusters[45]. The small clusters of PD-L2 may be insufficient to initiate PD-1-mediated inhibitory signals, which indicates that the inhibiting effect of the PD-1–PD-L pathway on autoimmunity will be decreased.

The frequencies of *PD-L2* 47103 C/T and 47139 T/C polymorphisms in Taiwanese were different from those of the Han Chinese and Japanese (NCBI Web resource: www:ncbi.nlm.nih.gov/SNP/). This discrepancy may be due to different genetic backgrounds. The associations of *PD-1* polymorphisms with lupus nephritis have been demonstrated in Sweden and Denmark[46,47], but not in the United States. However, a similar finding could not be found in *PD-L2* polymorphisms, which encode a ligand of PD-1. This may be due to different target genes and ethnic difference. Our study also revealed that the *PD-L1* and *PD-L2* polymorphisms were not associated with the severity of SLE, which was evaluated by the SLICC/ACR damage index.

PD-L2 47103 T may be associated with susceptibility to SLE in Taiwan. However, *PD-L1* polymorphisms are not related to the development of SLE.

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