CLINICAL STUDY

Association of an A/C single nucleotide polymorphism in programmed cell death-ligand 1 gene with Graves' disease in Japanese patients

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Abstract

Objective: Programmed cell death-1 (PD-1) and its ligands (PD-L1 and PD-L2) inhibit T-cell proliferation and activation. This inhibition down-regulates the immune responses. The association of a PD-L1 polymorphism with Graves' disease (GD) was studied.

Design: The association of an A/C polymorphism at position 8923 in PD-L1 intron 4 with GD was studied.

Patients: The study included 327 GD patients and 192 controls, of which 252 GD patients were followed over 5–10 years.

Measurements: PD-L1 intron 4 position 8923 A/C polymorphism was typed using the PCR-restriction fragment length polymorphism method.

Results: The A/C genotype frequencies were significantly different between GD patients and controls. The A/C and C/C frequencies were higher in GD patients than in controls. The A/A frequencies were lower in GD patients than in controls. C-allele frequency was higher in GD patients than in controls. A total of 252 GD patients were followed over 5–10 years; 200 had discontinued antithyroid drugs (ATD) while 52 continued to take ATD. Of these 200, 176 continued to be in remission and 24 had relapsed into hyperthyroidism. Significant differences in the duration of positive TBII, positive thyroid-stimulating antibodies, and ATD treatment were noted between the patients in remission and those that had relapsed. Significant differences in the A- and C-allele frequencies were noted between the two. The C-allele frequency was higher in GD patients who did not achieve remission than in those who achieved remission.

Conclusion: An A/C polymorphism at position 8923 in PD-L1 is associated with GD. The PD-L1 polymorphism plays a role in GD development. GD patients with the C allele at position 8923 in PD-L1 gene had difficulty in achieving remission.

European Journal of Endocrinology 158 817–822

Introduction

Graves' disease (GD) is a thyroid-specific autoimmune disorder associated with a T-lymphocyte abnormality (1). GD develops as a result of a complex interaction between genetic susceptibility genes and environmental factors (1, 2).

Programmed cell death 1 (PD-1) receptor and its ligands (PD-L1 and PD-L2) inhibit T-cell proliferation and activation (3). This inhibition down-regulates the immune responses (3). The PD-1 pathway is involved in the development of autoimmune diseases (4). PD-L1 and PD-L2 are ligands for PD-1 (5). PD-L1 is expressed on antigen-presenting cells such as dendritic cells and monocytes, on a subpopulation of T and B cells, and constitutively on parenchymal cells (6). PD-1 inhibits T-cell proliferation (7, 8). PD-1 and its ligands play a crucial role in the down-regulation of immune responses.

The alteration of the PD-1 and its ligand genes may contribute to the development of autoimmune diseases.

PD-1 gene polymorphisms have been reported to be associated with systemic lupus erythematosus (SLE) (9, 10), type 1 diabetes (T1D) (11, 12), rheumatoid arthritis (13), multiple sclerosis (MS) (14), and ankylosing spondylitis (AS) (15). A PD-L2 gene polymorphism is associated with susceptibility to SLE (16). Newby *et al.* (17) reported tag single nucleotide polymorphism (SNP) screening of the PD-1 gene for an association with GD.

A reduced expression or function of PD-L1 due to gene polymorphisms may induce an augmentation of the T- or B-cell responses. This augmentation of the T- or B-cell responses may lead to the development of autoimmune diseases. There are seven SNPs in the PD-L1 gene, which have so far been observed in the Japanese population (JSNPs database (<http://snp.ims.u-tokyo.ac.jp/index.html>)). Several gene polymorphisms have been reported in PD-L1 gene (12). However, no association between PD-L1 gene polymorphisms and autoimmune diseases has yet been reported. This study characterized the association between these PD-L1 gene polymorphisms

and GD. This is the first report to demonstrate an association between a PD-L1 gene polymorphism and GD. An A/C polymorphism at position 8923 in PD-L1 gene affects the GD development.

Methods

Subjects

Whole blood specimens with EDTA were obtained from the subjects. The subjects were recruited between 1 January 1993 and 31 December 2001. They were all Japanese. A total of 327 patients with GDs were studied (262 women and 65 men, aged 15–75 years). An A/C polymorphism at position 8923 in PD-L1 gene was analyzed in these 327 Graves’ patients. A total of 252 Graves’ patients were followed over 5–10 years. The other 75 Graves’ patients were not followed. In addition, the 192 normal control subjects were also observed (154 women and 38 men, aged 19–75 years). These 192 normal control subjects did not have a goiter. They also denied any personal or family histories of autoimmune thyroid diseases. They were negative for thyrotrophin (TSH) receptor antibodies (TRAb), thyroglobulin antibodies (TGAbs), and thyroid peroxidase antibodies (TPOAb). GD was diagnosed on the basis of histories and signs of hyperthyroidism with diffuse goiter and the laboratory findings, including elevated serum thyroxine (T₄) and tri-iodothyronine (T₃) concentrations, undetectable serum TSH levels, and positive TRAb (18, 19). TRAb was measured as TSH-binding inhibitory immunoglobulins (TBII) (20) and thyroid-stimulating antibodies (TSAb) (18). TBII was measured using a commercially available kit (RSR Ltd, Cardiff, UK). A receiver operating characteristic curve analysis (21) was conducted to evaluate the TBII and TSAb values for the GD diagnosis (20). The cutoff value for TBII was 10% (sensitivity 95.67% with 95% confidence limits (93.27–98.07%) and specificity 98.69% with 95% confidence limits (97.84–99.54%))

(20). TSAb was measured in the same manner as reported previously (18), using a commercially available kit (Yamasa, Chiba, Japan). The cutoff value for TSAb was 180% (sensitivity 96.14% with 95% confidence limits (94.64–97.64%) and specificity 98.62% with 95% confidence limits (97.87–99.37%)) (18).

Table 1 shows the clinical findings of the 252 Graves’ patients who had been followed for 5–10 years. After the diagnosis of GD was made, the patients were treated with methimazole (or propylthiouracil; antithyroid drugs (ATD)) at initial doses of 20–30 (or 200–300) mg/day. These doses were gradually reduced. They received the minimum doses of ATD necessary to maintain the normal levels of serum T₄, T₃, and TSH. The patients were seen every 2–4 weeks until their serum T₄ and T₃ concentrations had become normal. They were then followed every 4 weeks. TBII and TSAb were measured every 1–3 months. Of the 252 patients, 200 of them (group 1), who had had negative TBII and negative TSAb and normal serum levels of T₃, T₄, and TSH over the previous 1 year, had discontinued ATD, while 52 patients (group 2), who continued to have positive TBII and/or positive TSAb, continued to take ATD. The 200 patients (group 1), who had had negative TBII and negative TSAb and normal serum levels of T₃, T₄, and TSH over the previous 1 year, had all discontinued ATD. These patients were seen every 1–3 months. If the patients continued to be in a euthyroid state and to have negative TSAb and negative TBII at 1 year after ATD discontinuation, they were considered to be in remission (19). Of these 200 Graves’ patients in group 1, 176 patients were in remission (group 1A) while the other 24 patients relapsed into hyperthyroidism (group 1B). No significant differences in the gender ratio, the ages at diagnosis, and the titers of TBII and TSAb before treatment were noted between groups 1 and 2, or between groups 1A and 1B. Significant differences in the durations of positive TBII, positive TSAb, and ATD treatment were noted between groups 1A and 1B. Early disappearances of TBII and TSAb predict the remission (22). We studied the frequencies of AA, AC,

Table 1 Clinical findings of the 252 Graves’ patients.

Group (number of cases)	Women/men	Ages ^a	Before ATD treatment		Durations (years)		
			TBII (%)	TSAb (%)	+ TBII	+ TSAb	ATD treatment
Group 1 (200) ^b							
Group 1A (176) ^c	145/31	38.5 ± 16.5	49.8 ± 24.3	592 ± 196	2.8 ± 1.8	3.0 ± 1.8	4.0 ± 2.4
Group 1B (24) ^d	19/5	38.8 ± 17.5	50.1 ± 21.5	673 ± 215	5.3 ± 2.4	5.8 ± 2.9	6.9 ± 2.9
Group 2 (52) ^e	42/10	38.0 ± 19.5	56.2 ± 28.6	779 ± 395	Having positive TBII and/or TSAb Taking ATD		

Values are the means ± s.d. ATD, antithyroid drugs; TBII, TSH-binding inhibitory immunoglobulin; TSAb, thyroid stimulating antibody. No significant differences in the ages at diagnosis and the titers of TBII and TSAb before treatment were noted between groups 1 and 2, or between groups 1A and 1B. Significant differences were noted between groups 1A and 1B regarding the durations of positive TBII (*P* = 0.020), positive TSAb (*P* = 0.012), and ATD treatment (*P* = 0.018; Student’s *t*-test).

^aAges: ages at diagnosis.

^bTBII and TSAb had disappeared. They discontinued ATD.

^cThey continued to be in remission.

^dThey relapsed into hyperthyroidism.

^eTBII and/or TSAb persisted.

and CC genotypes and the A and C alleles of an A/C polymorphism at position 8923 in PD-L1 gene.

The study plan was reviewed and approved by the institutional review committee. Written informed consent was obtained from both the Graves' patients and the control subjects.

Genotyping

DNA was extracted from peripheral blood using a DNA purification kit (Qiagen). The PD-L1 intron 4 position 8923 A/C polymorphism was typed using the PCR–restriction fragment length polymorphism method (PCR–RFLP). The appropriate segment of the PD-L1 gene was amplified using specific primers (5'-AATGGCTTGTGTC-CAGAGATG-3' and 5'-GTACCACATGGAGTGGCTGC-3') using premix Taq (Takara, Shiga, Japan). PCR was performed by initial denaturation for 10 min at 95 °C, annealing for 30 s at 60 °C, extension for 3 min at 72 °C, denaturation for 30 s at 95 °C (37 cycles), and a final extension for 10 min at 72 °C. The amplified products (553 bp) digested with restriction enzyme Ban II (Takara) and then analyzed on 3.0% NuSiev GTG agarose (FMC BioProducts, Philadelphia, PA, USA) gels. The PCR fragments with a C allele at position 8923 were cut into three fragments (97, 130, and 326 bp), whereas the fragments with A at the same position were cut into two fragments, namely they had the restriction site only at 97 bp while also developing a 456 bp band.

Statistical analysis

Statistical analyses were done using Fisher's exact test or Student's *t*-test. A value of $P < 0.05$ was considered to be significantly different. Power and Hardy–Weinberg equilibrium calculations were performed using Excel (Microsoft Office Excel, 2003).

Results

Table 2 shows A/C SNP at position 8923 in the PD-L1 gene in the 327 Graves' patients and the 192 normal

control subjects (controls). Significant differences in the A/C genotype frequencies were noted between Graves' patients and controls. The A/C and C/C genotype frequencies were higher in Graves' patients than in controls. The A/A genotype frequencies were lower in Graves' patients than in controls. The C-allele frequency in Graves' patients (8.3%) was also higher than that in controls (3.1%). Significant differences in the genotype frequencies ($P = 0.0022$) and the allele frequencies ($P = 0.0009$) were noted between Graves' patients and controls, producing an odds ratio (OR) = 2.79 (95% confidence intervals (CI) = 1.47–5.28).

Table 3 shows genotype and allele frequencies of A/C SNP PD-L1 at position 8923 in the 252 Graves' patients, who had been followed over 5–10 years. The A/C and C/C frequencies were 11.9 and 0.0% respectively in the 176 patients who continued to be in remission (group 1A), but the A/C and C/C genotype frequencies were 16.7 and 8.3% respectively in the other 24 patients who had relapsed into hyperthyroidism (group 1B). Significant differences in the genotype frequencies ($P = 0.0102$) were noted between the 176 (group 1A) and the other 24 (group 1B) patients. The C-allele frequency was 6.0% in the 176 (group 1A), but it was 16.7% in the other 24 (group 1B) patients. Significant differences in the allele frequencies ($P = 0.0143$) were noted between the 176 (group 1A) and the other 24 (group 1B) patients. Significant differences in the A- and C-allele frequencies were noted between groups 1A and 1B, between groups 1 and 2, and between groups 1A and 2. However, no significant differences in the A- and C-allele frequencies were noted between groups 1B and 2.

Discussion

These data demonstrate the association of a PD-L1 gene polymorphism with GD. An A/C polymorphism at position 8923 in PD-L1 gene is associated with the GD development and also with a relapse of Graves' hyperthyroidism.

GD develops as a result of a complex interaction between genetic susceptibility genes and environmental factors (23, 24). A model analysis suggests that the GD

Table 2 Genotype and allele frequencies of A/C SNP PD-L1 gene at position 8923 in 327 Graves' patients (Graves') and 192 normal control subjects (controls).

	Graves' <i>n</i> (%)	Controls <i>n</i> (%)	<i>P</i>	OR	95% CI
Genotype					
A/A	277 (84.7)	181 (94.3)	0.0022	2.79	1.47–5.28
A/C	46 (14.1)	10 (5.2)			
C/C	4 (1.2)	1 (0.5)			
Allele					
A	600 (91.7)	372 (96.9)	0.0009	2.79	1.47–5.28
C	54 (8.3)	12 (3.1)			

PD-L1, programmed cell death-ligand 1; SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence intervals. Statistically significant differences were noted between Graves' patients and normal control subjects in the genotype frequencies ($P = 0.0022$) and allele frequencies ($P = 0.0009$). Statistical analyses were done using Fisher's exact test.

Table 3 Genotype and allele frequencies of A/C SNP PD-L1 gene at position 8923 in the 252 Graves' patients.

	Group 1 (200)		Group 2 (52)
	A. Remission (176) n (%)	B. Relapsed (24) n (%)	
Genotype			
A/A	155 (88.1)	18 (75.0)	38 (73.0)
A/C	21 (11.9)	4 (16.7)	12 (23.1)
C/C	0 (0.0)	2 (8.3)	2 (3.8)
Allele			
A	331 (94.0)	40 (83.3)	88 (84.6)
C	21 (6.0)	8 (16.7)	16 (15.4)

PD-L1, programmed cell death-ligand 1; SNP, single nucleotide polymorphism; ATD, antithyroid drugs. Significant differences in A- and C-allele frequencies were noted between groups 1A and 1B ($P=0.0143$), between groups 1 and 2 ($P=0.0187$), and between groups 1A and 2 ($P=0.0037$; Fisher's exact test). However, no significant differences in A, the C-allele frequencies were observed between groups 1B and 2 ($P=0.8154$; Fisher's exact test).

patients have genetic susceptibility (25). The putative susceptibility genes for GD are immune-modifying genes (e.g., major histocompatibility complex (MHC) (24, 25), MHC class 1 chain-related gene A (MICA) (26), cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) (27), CD40 (28), and butyrophilin-like protein (BTLN2) that is likely due to a linkage disequilibrium with MHC class II (29)), thyroid-specific genes (e.g., TG (30) and TSH receptor (TSHR) (31)), cytokine-related genes (e.g., interleukin-1 receptor antagonist (IL-1RA) (32), IL-4 (33), and tumor necrosis factor- α (TNF- α) (34)). A recent study has shown that small effects within a PD-1 gene may contribute to the GD development (17).

Programmed death-1 (PD-1; a CD28 homologue) and its ligands (PD-L1 and PD-L2; homologous to B7) constitute an inhibitory pathway of T-cell co-stimulation. PD-1/PD-L1 interactions play a critical role in the positive selection of thymocytes while also playing a role in shaping the T-cell repertoire (35). PD-L1 is expressed on activated T cells, B cells, monocytes, dendritic cells, and on non-hematopoietic cells (35–41). PD-1–PD-L1 interaction has been suggested to play a central role in the regulation of induction and progression of autoimmune diabetes in NOD mice (42). PD-1 $-/-$ and PD-L1 $-/-$ mice developed more severe EAE than wild-type and PD-L2 $-/-$ mice (43). PD-1–PD-L1 interaction attenuates T-cell responses. A reduction of the PD-1/PD-L1 function (or expression) augments the T-cell responses, and it may also induce autoimmune diseases.

PD-1 gene polymorphisms have been reported to be associated with SLE (9, 10), AS (15), MS (14), T1D (12), and GD (17). The genotype frequency of PD-L2 47103 T/T, in comparison with 47103 C/C, has been reported to significantly increase in patients with SLE (16). A PD-L1 gene polymorphism has not been studied yet. This is the first report to demonstrate that a PD-L1 gene polymorphism is associated with autoimmune diseases. The C-allele frequency at position 8923 in the PD-L1 gene was higher in the GD patients than in the controls.

Graves' patients with a C allele at position 8923 in the PD-L1 gene had difficulty in achieving remission. An A/C polymorphism at position 8923 in the PD-L1 gene is associated with GD development and its remission.

This A/C polymorphism at position 8923 in the PD-L1 gene occurs in intron 4. It does not affect an amino acid substitution. One possible explanation is that the polymorphism is near or within transcriptional factor-binding sites (12), and it may also modify the binding affinity of transcriptional factors. This may influence the PD-L1 gene expression. It is also possible that position 8923 A/C SNP may affect the PD-L1 function.

There are seven SNPs in the PD-L1 gene in the Japanese population. Six SNPs are in introns and one SNP is in 3'-UTR in the PD-L1 gene. The A/C polymorphism at position 8923 in the PD-L1 gene is associated with the GD development and its relapse. The other six SNPs will be analyzed.

Several GD-susceptibility genes contribute to the GD development with high ORs. For example, the development of GD could be due to the genetic effects of MHC such as DRB1*03 with OR values 2.3–3.4 (44). The genotype A5.1/A5.1 in MICA gene occurred more frequently in patients with GD than in the controls (OR 2.0) (45). The A49G and CT60 polymorphisms of CTLA-4 were significant in associations with GD (OR 1.49 and 1.45 respectively) (46). The CC+CT genotypes of the CD40-E1SNP were associated with the increased risk for GD (OR 1.9) (47). For TG gene, the three SNPs of the exons 10–12 in combination with the exon 33 SNP were strongly associated with GD with ORs 2.44–2.58 (30). In addition, the allele frequencies of 1031C and -863A of TNF- α gene in the GD patients with evident ophthalmopathy were significantly greater than those with no or mild ophthalmopathy (ORs 2.94 and 2.30 respectively) (34). Some genes may affect the GD development with low ORs (17). The rs2076530 SNP in BTLN2 gene and +2375 SNP in PD-1 gene were associated with the development of GD, but these genetic effects were small (ORs 1.32 and 1.14 respectively) (17, 29). Our PD-L1 gene polymorphism at 8923 contributes to the GD development with a high OR of 2.79. It is still unclear whether this PD-L1 gene polymorphism strongly contributes to the GD development, because the number of patients and controls in our study was small and the allele frequency at position 8923 in the PD-L1 gene was minor. Occasionally, several factors have been shown to induce high ORs in similar studies with disease susceptibility genes. For example, studies with a small number of patients may generate high ORs. The OR of the development of GD with MHC gene is 2.3–3.4 in 135 GD patients (44), while that with MICA gene is 2.0 in 129 GD patients (45), that with CD40 gene is 1.9 in 301 GD patients (47), that with TG gene is 2.44–2.58 in 186 GD patients (30), and that with TNF- α gene is 2.94 in 173 GD patients (34). On the other hand, the OR of the development of GD with CTLA-4 gene is 1.49

in 22 038 GD patients (46), that with BTNL-2 gene is 1.32 in 1658 GD patients (29), and that with PD-1 gene is 1.14 in 4130 GD patients (17). We performed the power calculations with the distribution of the PD-L1 gene polymorphism in the 327 GD patients and the 192 controls. The power was therefore 24% to detect an OR=1.5 and $P=0.05$. Our PD-L1 gene polymorphism at 8923 contributes to the GD development with an OR of 2.79. This OR of 2.79 seems to be high. The minor frequency of the C allele may generate this high OR. The C-allele frequency in the Graves' patients (8.3%) was higher than that in the controls (3.1%), the difference being 5.2%. This could thus have produced the erroneously high OR of 2.79. There are some limitations in our study.

We describe that A/C SNP at 8923 might be one of the candidate gene polymorphisms for GD. Further studies will be required to determine a clear association of the PD-L1 gene polymorphism with the GD development in large numbers of patients, as well as patients with other different genetic backgrounds.

Disclosure

We have no conflict of interest that would prejudice its impartiality.

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Received 12 February 2008

Accepted 2 March 2008