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Analysis of HLA class II genes associated with susceptibility to type 1 diabetes in Japanese patients with autoimmune thyroid disease

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Objective: Type 1 diabetes (T1D) is an organ-specific autoimmune disease triggered by both genetic and environmental factors. Adult-onset T1D in Japan is frequently associated with autoimmune thyroid disease (AITD), and more than half of the T1D cases are preceded by AITD. We investigated whether the human leukocyte antigen (HLA) class II (DR/DQ) gene, the major genetic risk factor for T1D, is useful in identifying individuals at high risk of developing T1D among Japanese AITD patients.
Design/Patients: We genotyped the HLA class II (DR/DQ) gene in 82 patients with AITD complicated with T1D (AITD+T1D), 131 AITD patients without T1D (AITDw/oT1D), and 222 healthy subjects as controls.
Results: Compared to the controls, the AITD+T1D group had a significantly higher rate of the T1D-susceptible haplotype *DRB1*0405-DQB1*0401*, but the AITDw/oT1D group did not. Compared to the controls, the T1D-protective haplotypes *DRB1*1501-DQB1*0602/DRB1*1502-DQB1*0601* were significantly less frequent in the AITD+T1D patients but not in the AITDw/oT1D patients. In genotypes combining the *DRB1-DQB1* haplotypes susceptible to T1D, only DR4/DR8 was significantly more frequent compared to the controls. We classified the haplotypes into three types (susceptible [S], protective [P] and neutral [N]) to examine their association with T1D development. Compared to the controls, the genotypes S/S and N/P were observed more and less frequently, respectively, in AITD+T1D. No difference in the frequency of those genotypes between AITDw/oT1D and controls was observed.
Conclusions: The risk of the future development of T1D in Japanese patients with AITD could be stratified by an analysis of combinations of HLA *DRB1-DQB1* haplotypes.

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Key words: HLA class Ⅱ, type1 diabetes (T1D), autoimmune thyroid disease (AITD)

Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease in which the body's immune system destroys insulin-producing β-cells in the pancreas, leading to a loss of insulin secretion and hyperglycemia[1,2]. T1D develops as a consequence of a combination of a genetic predisposition, largely unknown environmental factors, and stochastic events. It is certain that both genetic and environmental factors contribute to the risk of developing T1D[3]. Due to the progress in human genetics, >50 susceptibility loci that contribute to the likelihood of developing T1D have been identified[4], and it is now known that the major histocompatibility complex region encoding human leukocyte antigen (HLA) DR-DQ on chromosome 6p21 contributes approx. 50% of the genetic risk[5].

In Europe and the United States, researchers have investigated the genetic risk of T1D in patients followed up from birth until clinical diagnosis among newborn children who were born in families with T1D or who have high-risk HLA-haplotypes (DR3-DQ2 and/or DR4-DQ8)[6]. In Japan, however, it is difficult to conduct such a cohort study in pre-diabetic subjects since the incidence of T1D in children is much lower

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than in Europe and the U.S.[7].

Adult-onset T1D with slowly-progressive subtypes is relatively common in Japan[8], and it is commonly associated with autoimmune thyroid disease (AITD) including Graves' disease (GD) and Hashimoto thyroiditis (HT)[9,10]. Based on a fact-finding survey of a large number of Japanese adults (health check examinees), the prevalence of the thyroid autoantibodies including thyroglobulin (Tg) antibody and/or thyroid peroxidase (TPO) antibody was estimated as 14.4% for men, 24.7% for women, and 21.7% overall[11]. Approximately 24.03 million adults in Japan have some type of autoimmune thyroid disease (AITD), among whom roughly 7.92 million have thyroid dysfunction; it is estimated that the majority of the cases of overt thyroid dysfunction are caused by AITD[12]. We reported that 60% of individual who developed both T1D and GD in their lifetime developed GD before the onset of T1D[13]. It could be clinically significant to screen patients diagnosed with AITD in order to identify individuals who are at high risk of developing T1D and for the tracking of the progression of diabetes; these actions could help elucidate the pathophysiology of Japanese-specific adult T1D.

The HLA class II *DRB1* and *DQB1* alleles, or those haplotypes, are major susceptibility genes to T1D in various ethnic groups including Japanese[14]. The T1D-susceptible HLA haplotypes in Caucasians, i.e., DR4 (e.g., *DRB1*0401–DQB1*0302* and *DRB1*0301–DQB1*0201*), are rarely detected in Japanese patients with T1D. Instead, *DRB1*0405–DQB1*0405*, *DRB1*0802–DQB1*0302*, and *DRB1*0901–DQB1*0302* are known as the major haplotypes of susceptibility for T1D in Japanese[15]. The protective haplotypes for T1D in Japanese are *DRB1*1502–DQB1*0601* in addition to *DRB1*1501–DQB1*0602*, which is also protective in Caucasians[15].

In this study, we analyzed the HLA class II (DR/DQ) haplotype and genotype in AITD patients with T1D and those without T1D to determine whether the HLA gene is useful in identifying Japanese AITD patients who are at high risk of developing T1D.

Patients and Methods

Disease definition

The diagnosis of T1D is based on the criteria and classification of the Japan Diabetes Society[13]. We recruited patients with type 1A diabetes developed by an autoimmune mechanism to pancreatic β-cells who were characterized by having one or more anti-islet autoantibodies including antibodies (Abs) to glutamic acid decarboxylase (GAD), insulinoma-associated antigen-2 (IA-2), insulin, and zinc transporter 8 (ZnT8). Patients with idiopathic T1D (i.e., type 1B diabetes) who were not proven to have any anti-islet autoantibodies were excluded from the study.

Cases of AITD including GD and HT were diagnosed according to the criteria of the Japan Thyroid Association[16]. GD was defined as a history of primary hyperthyroidism with a positivity of thyroid stimulating hormone (TSH) receptor Abs. HT was defined as having diffuse goiter and/or primary hypothyroidism with positive Abs to TPO and/or Tg. Patients who were positive only for TPOAbs/TgAbs without a definitive medical record of hypothyroidism or goiter formation were excluded from the study. Patient who had been diagnosed with both GD and HT were classified as patients with GD.

Patients

We identified 213 Japanese patients with AITD diagnosed at Nagasaki University Hospital from 1983 to 2001. We divided the 213 patients with AITD into two groups depending on whether they were complicated with T1D (AITD+T1D, n=82) or not (AITDw/oT1D, n=131). As a control group, we included 222 healthy subjects who had developed neither AITD nor diabetes. Informed consent was obtained from all participants in the study, which was approved by the Ethical Committee of Nagasaki University Hospital (approval no. 10042864).

Autoantibody assay

The GAD Abs, IA-2Abs, TgAbs, and TPOAbs were measured using a commercially available radioimmunoassay (RIA) kit (Cosmic Co., Tokyo). Insulin autoantibodies and TSH receptor Abs were measured using an RIA kit (Yamasa Co., Chiba, Japan). ZnT8Abs were measured by radioligand binding assay as described[17].

Genotyping of HLA-DRB1 and -DQB1

The HLA-*DRB1* and -*DQB1* alleles were genotyped by polymerase chain reaction-restriction fragment length polymorphism methods as reported[14]. Haplotypes were determined based on the most probable haplotypes according to the linkage disequilibria in the Japanese population[18].

Statistical analysis

The frequencies of each HLA *DRB1-DQB1* haplotype in the AITD patients with and without T1D were compared to the healthy controls using the odds ratio (OR) and its 95% confidence interval (CI). Fisher's exact test was used to study the differences in the distribution of the HLA *DRB1-DQB1* haplotypes. A p-value <0.05 was considered significant. In the multiple comparisons, the Bonferroni correction was used to examine the significance between the groups. All the data was analyzed using the R software (ver. 4.1.0).

Results

Clinical characteristics of the AITD patients with/without T1D

The clinical characteristics of the 213 patients with AITD included in the study are summarized in Table 1. Of the 213 patients with AITD, 82 patients developed T1D and 131 patients did not. The female-to-male ratio was not significantly different between the AITD patients with T1D and those without T1D (p=0.069). The ratio of GD to HT was significantly lower in the AITD+T1D group compared to the AITDw/oT1D group (p<0.0001).

The frequencies of the HLA DRB1-DQB1 haplotype in AITD with/without T1D

The frequencies of the HLA *DRB1-DQB1* haplotypes in the AITD patients with and without T1D are listed in Table 2. We investigated whether these two subtypes have different haplotypes profiles by comparing them with the healthy control subjects. The *DRB1*0405-DQB1*0401* haplotype, which is known as one of the major haplotypes susceptible to T1D, was significantly more frequent in the AITD+T1D patients compared to the controls (OR 3.08, p<0.0001). Another T1D-susceptible haplotype, *DRB1*0802-DQB1*0302*, was more frequent in the AITD+T1D group compared to the control group (OR 2.80, p=0.0325) but the difference was not significant after Bonferroni correction. The frequency of *DRB1*0901-DQB1*0303*, which has a susceptibility to T1D, was comparable between the AITD+T1D patients and the controls. The major T1D-protective haplotypes *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601* were significantly less frequent in the AITD+T1D group compared to the controls (OR 0.00, p=0.0002 and OR 0.19, p=0.0002, respectively).

The AITD patients without T1D showed a higher frequency of both *DRB1*0405-DQB1*0401* and *DRB1*1602-DQB1*0502* compared to the controls, but the differences were not significant after Bonferroni correction.

The frequencies of the HLA DRB1-DQB1 genotype in AITD with/without T1D

We studied the frequencies of the combinations among three T1D-susceptible haplotypes, i.e., DR4 (*DRB1*0405-DQB1*0401*), DR8 (*DRB1*0802-DQB1*0302*) and DR9 (*DRB1*0901-DQB1*0303*) in the AITD patients with T1D and without T1D (Table 3). The genotypes of DR4/4, 4/8, and 4/9 in the AITD+T1D group were significantly more frequent than among the controls, but the significance remained only for DR4/8 (p=0.0003) after Bonferroni correction. The combination with DRXs (DRX/X) was significantly less frequent in the AITD+T1D patients compared to the controls (OR 0.23, p<0.0001).

In contrast, no significant difference was observed in the frequency of any genotypes between the AITD patients without T1D and the controls.

Table 1. Clinical characteristics of the AITD patients with and without T1D

	AITD patients		p-value
	with T1D	without T1D	
n	82	131	
Sex (male/female)	20/62	19/112	0.069
Type of AITD (GD/HT)	31/51	90/41	<0.0001

AITD: autoimmune thyroid disease, T1D: type 1 diabetes, GD: Graves' disease, HT: Hashimoto thyroiditis.

Table 2. The HLA *DRB1-DQB1* haplotypes in AITD patients with and without T1D

DRB1-DQB1	AITD with T1D (n=164)		AITD without T1D (n=262)		Controls (n=444)		AITD + T1D vs. Controls			AITDw/oT1D vs. Controls		
	n	%	n	%	n	%	p	OR	(95%CI)	p	OR	(95%CI)
**0101–*0501*	3	(1.8)	13	(5.0)	25	(5.6)	n.s.	0.31	(0.06–1.05)	n.s.	0.88	(0.40–1.81)
**0301–*0201*	1	(0.6)	7	(2.7)	4	(0.9)	n.s.	0.68	(0.01–6.89)	n.s.	3.01	(0.76–14.18)
**0403–*0302*	6	(3.7)	2	(0.8)	10	(2.3)	n.s.	1.65	(0.48–5.10)	n.s.	0.33	(0.04–1.59)
**0405–*0401*	52	(31.7)	52	(19.8)	58	(13.1)	<0.0001	3.08	(1.96–4.85)	0.0182 [a]	1.65	(1.07–2.53)
**0406–*0302*	4	(2.4)	4	(1.5)	16	(3.6)	n.s.	0.67	(0.16–2.12)	n.s.	0.42	(0.10–1.31)
**0802–*0302*	9	(5.5)	3	(1.1)	9	(2.0)	0.0325 [a]	2.80	(0.97–8.12)	n.s.	0.56	(0.10–2.27)
**0803–*0601*	12	(7.3)	24	(9.2)	35	(7.9)	n.s.	0.92	(0.42–1.88)	n.s.	1.18	(0.65–2.09)
**0901–*0303*	39	(23.8)	43	(16.4)	83	(18.7)	n.s.	1.36	(0.86–2.13)	n.s.	0.85	(0.56–1.30)
**1101–*0301*	2	(1.2)	2	(0.8)	11	(2.5)	n.s.	0.49	(0.05–2.26)	n.s.	0.30	(0.03–1.41)
**1201–*0301*	2	(1.2)	8	(3.1)	9	(2.0)	n.s.	0.60	(0.06–2.93)	n.s.	1.52	(0.50–4.51)
**1302–*0604*	11	(6.7)	10	(3.8)	27	(6.1)	n.s.	1.11	(0.48–2.38)	n.s.	0.61	(0.26–1.33)
**1501–*0602*	0	(0.0)	21	(8.0)	28	(6.3)	0.0002	0.00	(0.00–0.36)	n.s.	1.29	(0.68–2.42)
**1502–*0601*	4	(2.4)	20	(7.6)	51	(11.5)	0.0002	0.19	(0.05–0.54)	n.s.	0.63	(0.35–1.12)
**1602–*0502*	1	(0.6)	7	(2.7)	1	(0.2)	n.s.	2.71	(0.03–213.44)	0.0050 [a]	12.12	(1.54–547.80)
Others	18	(11.0)	46	(17.6)	77	(17.3)	n.s.	0.59	(0.32–1.03)	n.s.	1.02	(0.66–1.54)

"Others" includes rare haplotypes whose total frequencies in each group were <2.5%.

[a] Not significant after a Bonferroni multiple adjustment (the threshold for statistical significance is p<0.05/15). n.s.: not significant.

The frequencies of the combination of HLA DRB1-DQB1 haplotypes susceptible/neutral/protective to develop T1D in AITD patients with/without T1D

As reported[19], we classified those HLA-*DRB1-DQB1* haplotypes into the T1D-susceptible/neutral/protective haplotypes: **0405-*0401*, **0802-*0302*, and **0901-*0303* were classified as susceptible haplotypes (S); **1501-*0602* and **1502-*0601* were classified as protective haplotypes (P), and the others were classified as neutral haplotypes (N). The frequencies of the combinations of these haplotypes in the AITD patients with/without T1D are provided in Table 4.

The genotype of S/S was significantly more frequent in the AITD+T1D group compared to the controls (OR 5.24, p<0.0001). The N/P genotype was not found in the AITD+T1D group but was detected in 19.4% of the controls (p<0.0001). The frequency of the genotype S/Y, which indicates the combination with S and any allele including S, was significantly higher in the AITD+T1D patients compared to the controls (OR 3.69, p<0.0001). Conversely, the genotype P/Y, which indicates the combination with P and any allele including P, was significantly lower in the AITD+T1D group than the controls (OR 0.10, p<0.0001). We did not observe any differences in the frequencies of all of the *DRB1-DQB1* genotypes between the AITD patients without T1D and the healthy controls.

Table 3. The HLA *DRB1-DQB1* genotypes in the AITD patients with and without T1D

DRB1-DQB1	AITD with T1D (n=82)		AITD without T1D (n=131)		Controls (n=222)		AITD + T1D vs. Controls			AITDw/oT1D vs. Controls		
	n	%	n	%	n	%	p	OR	(95%CI)	p	OR	(95%CI)
DR4/4	6	(7.3)	6	(4.6)	2	(0.9)	0.0057[a]	8.61	(1.50–89.10)	n.s.	5.25	(0.92–53.97)
DR4/9	11	(13.4)	5	(3.8)	9	(4.1)	0.0071[a]	3.65	(1.31–10.41)	n.s.	0.94	(0.24–3.20)
DR4/8	6	(7.3)	1	(0.8)	0	(0.0)	0.0003	n.a.	n.a.	n.s.	n.a.	n.a.
DR9/9	8	(9.8)	8	(6.1)	10	(4.5)	n.s.	2.28	(0.75–6.71)	n.s.	1.38	(0.46–3.99)
DR9/8	1	(1.2)	0	(0.0)	2	(0.9)	n.s.	1.36	(0.02–26.38)	n.s.	0.0	n.a.
DR4/X	23	(28.0)	34	(26.0)	45	(20.3)	n.s.	1.53	(0.81–2.84)	n.s.	1.38	(0.80–2.36)
DR9/X	11	(13.4)	22	(16.8)	43	(19.4)	n.s.	0.65	(0.28–1.36)	n.s.	0.84	(0.45–1.53)
DR8/X	2	(2.4)	2	(1.5)	5	(2.3)	n.s.	1.08	(0.10–6.79)	n.s.	0.67	(0.06–4.19)
DRX/X	14	(17.1)	53	(40.5)	105	(47.3)	<0.0001	0.23	(0.11–0.44)	n.s.	0.76	(0.48–1.20)

DR4, DR8, and DR9 indicate *DRB1*0405-DQB1*0401* haplotype, *DRB1*0802-DQB1*0302* haplotype, and *DRB1*0901-DQB1*0303* haplotype, respectively. X indicates haplotypes other than *DR4, DR8* and *DR9*. [a] Not significant after a Bonferroni multiple adjustment (threshold for statistical significance: p<0.05/9). n.s., not significant; n.a., not applicable.

Table 4. The frequencies of the HLA *DRB1-DQB1* genotypes susceptible/neutral/protective to develop T1D in the AITD patients with and without T1D

DRB1-DQB1	AITD with T1D (n=82)		AITD without T1D (n=131)		Controls (n=222)		AITD + T1D vs. Controls			AITDw/oT1D vs. Controls		
	n	%	n	%	n	%	p	OR	(95%CI)	p	OR	(95%CI)
S/S	32	(39.0)	20	(15.3)	24	(10.8)	<0.0001	5.24	(2.73–10.22)	n.s.	1.48	(0.74–2.95)
S/N	32	(39.0)	44	(33.6)	75	(33.8)	n.s.	1.25	(0.71–2.18)	n.s.	0.99	(0.61–1.60)
S/P	4	(4.9)	14	(10.7)	27	(12.2)	n.s.	0.37	(0.09–1.12)	n.s.	0.86	(0.40–1.79)
N/N	14	(17.1)	31	(23.7)	49	(22.1)	n.s.	0.73	(0.35–1.45)	n.s.	1.09	(0.63–1.88)
N/P	0	(0.0)	17	(13.0)	43	(19.4)	<0.0001	0.0	n.a.	n.s.	0.62	(0.32–1.18)
P/P	0	(0.0)	5	(3.8)	4	(1.8)	n.s.	0.0	n.a.	n.s.	2.16	(0.46–11.08)
S/Y	68	(82.9)	78	(59.5)	126	(56.8)	<0.0001	3.69	(1.91–7.54)	n.s.	1.12	(0.71–1.78)
P/Y	4	(4.9)	36	(27.5)	74	(33.3)	<0.0001	0.10	(0.03–0.29)	n.s.	0.76	(0.46–1.25)

S: Susceptible haplotypes for T1D including *DRB1*0405-DQB1*0401, DRB1*0802-DQB1*0302* and *DRB1*0901-DQB1*0303.*

P: Protective haplotypes against T1D including *DRB1*1501-DQB1*0602, DRB1*1502-DQB1*0601.*

N: Neutral haplotypes other than susceptible and protective haplotypes. Y: any haplotype (S+N+P).

[a] Not significant after a Bonferroni multiple adjustment (threshold for statistical significance, p<0.05/8).

Discussion

The present results demonstrated that Japanese AITD patients with T1D showed a positive association with *DRB1*0405-DQB1*0401* and a negative association with either *DRB1*1501-DQB1*0602 or DRB1*1502-DQB1*0601*, but the AITD patients without T1D did not. These results suggest that the HLA genes in AITD patients may be associated with the risk of adult T1D in Japan.

As shown in Table 3, among the combinations of disease susceptibility haplotypes for T1D (i.e., DR4, DR8 and DR9), only the genotype DR4/8 was associated with the development of T1D in Japanese patients with AITD. However, it has been reported that the frequency of the HLA DR4/8 genotype is very low in the Japanese population[18]. It is difficult to distinguish AITD patients at high risk of developing T1D from all patients with AITD. We therefore further classified the HLA haplotypes into those that are susceptible (S), protective (P), or neutral (N) to T1D. We analyzed the involvement of the combination of genotypes in disease susceptibility to T1D, and as shown in Table 4, the combination of susceptible haplotypes (S/S) was observed in the AITD patients with T1D at a frequency that was approx. fivefold that of the controls. Since heterozygotes showed no disease susceptibility, this result might indicate the possibility of disease susceptibility in the recessive model. However, the combination with the allele (S) and any haplotype (Y) (S/Y=S/S+S/N+S/P) showed a significant association with the development of T1D. It is difficult to determine the association with those genetic forms due to the small number of patients examined herein. Further study with a large number of cases should be considered.

The genotype N/P, a combination with a T1D-protective and T1D-neutral haplotype, showed a strong resistance to the development of T1D among the AITD patients. The combinations with the allele (P) and any allele (Y) (P/Y=P/S+N/P+P/P) also showed a strong resistance to the development of T1D, suggesting that the genetic form is a dominant model. Homozygotes of resistant haplotypes (P/P) or combinations of susceptible and resistant haplotypes (S/P) did not show significant disease resistance. This might be because the sample size was too small to meet the significance level.

Despite the improvement in the management of T1D with exogenous insulin over the past several decades, most patients with T1D cannot achieve adequate glycemic control[20]. Clinical and preclinical trials have suggested strategies to prevent the clinical development of T1D in asymptomatic individuals at high risk of T1D; however, the outcomes of the trials have not achieved their primary endpoint (T1D prevention or delay)[21]. The landscape for the prevention of T1D is dramatically changing[22]. A multicenter interventional trial demonstrated that the clinical onset of T1D was delayed by treatment with a monoclonal antibody to CD3 (teplizumab) in individuals at high risk for T1D who have the HLA DR4-DQ8 haplotype[23]. Another clinical trial demonstrated that endogenous insulin production was preserved by treatment with specific immunotherapy using alum-formulated GAD65 (GAD-alum) in individuals with recent-onset T1D who have the DR3-DQ2 haplotype[24]. Thus, the HLA types were associated with the efficacy of these immunotherapies, in addition to immunopathology or disease susceptibility in T1D[25]. The analysis of the combination of the HLA genes and multiple islet-related autoantibodies could become of great importance for the selection of eligible subjects for promising immunotherapy among individuals who are at high risk of developing T1D in the near future[26].

Our present findings demonstrated that the HLA genes in Japanese patients with AITD patients are associated with the risk of the development of adult T1D. However, the odds ratio was 5.24, which may be insufficient to stratify the risk of developing T1D or to serve as a criterion for selecting patients for T1D-preventive treatment. A genetic risk score (GRS) incorporating T1D-associated single nucleotide polymorphisms (SNPs) in HLA and non-HLA regions has been tested to discriminate T1D from type 2 diabetes and to predict the subsequent onset of T1D in subjects at high risk of T1D[27,28]. It is necessary to develop a Japanese-specific GRS that combines disease-susceptibility genes in the HLA and non-HLA domains in order to accurately assess the risk of T1D among AITD patients.

There are several limitations to this study. The estimated detection power is limited due to the small sample size of the target. The ratios of HT and GD in our patient series do not reflect those in the whole Japanese population; this may be because GD patients are predominantly referred to specialized facilities such as university hospitals. More real-world epidemiological validation is needed in the future.

Conclusion

The results of our study suggest that among Japanese patients with AITD, the risk of the development of T1D could be stratified by a combination analysis of HLA *DRB1-DQB1* haplotypes. In Japan, unlike in Europe and the U.S., there may be a need to identify individuals who are at high risk of developing T1D among adults with AITD in order to consider the prevention of T1D with immunotherapies according to their risk.

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