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Identification of susceptibility loci for autoimmune thyroid disease to 5q31–q33 and Hashimoto's thyroiditis to 8q23–q24 by multipoint affected sib-pair linkage analysis in Japanese

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Autoimmune thyroid disease (AITD), including Graves' disease (GD) and Hashimoto's thyroiditis (HT), is caused by multiple genetic and environmental factors. The clinical and immunological features of GD and HT are distinct; however, there are multiplex families with both GD and HT, and cases in which GD evolves into HT. Thus, there may be specific susceptibility loci for GD or HT, and common loci controlling the susceptibility to both GD and HT may exist. A genome-wide analysis of data on 123 Japanese sib-pairs affected with AITD was made in which GD- or HT-affected sib-pairs (ASPs) were studied to detect GD- or HT-specific susceptibility loci, and all AITD-ASPs were used to detect AITD-common susceptibility loci. Our study revealed 19 regions on 14 chromosomes (1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 15, 18 and 22) where the multipoint maximum LOD score (MLS) was >1. Especially, chromosome 5q31–q33 yielded suggestive evidence for linkage to AITD as a whole, with an MLS of 3.14 at D5S436, and chromosome 8q23–q24 yielded suggestive evidence for linkage to HT, with an MLS of 3.77 at D8S272. These observations suggest the presence of an AITD susceptibility locus at 5q31–q33 and a HT susceptibility locus at 8q23–q24.

INTRODUCTION

Autoimmune thyroid disease (AITD), including Graves' disease and Hashimoto's thyroiditis (HT), is caused by immune responses related to the thyroid gland. Twin studies and familial aggregation showed that AITD is a complex disease with genetic factors (1,2). Furthermore, GD and HT cluster in a family (3), in identical twins and triplets (4), and those in whom GD evolved into HT (5); thus, there may exist common susceptibility loci shared by GD and HT. On the other hand, there are clear differences in the clinical and immunological features between GD and HT, and statistical association of specific human leukocyte antigen (HLA) alleles with GD and HT (6–8), respectively; hence specific susceptibility loci for GD or HT may exist.

Several susceptibility loci, including GD-1 (14q31), GD-2 (20q11.2) and GD-3 (Xq21) for GD; AITD-1 (6p) for AITD; and HT-1 (13q32) and HT-2 (12q22) for HT, were noted in a linkage analysis using multiplex families (9–14). Non-parametric linkage analysis using affected sib-pairs (ASPs) showed evidence for linkage of HLA, the cytotoxic T lymphocyte-associated-4 (CTLA-4) and IDDM6 to GD (15,16). In addition, significant associations were noted between AITD and specific HLA alleles (6–8,17–19), and between AITD and CTLA-4 gene polymorphism (20–28).

Here, we carried out a genome-wide screening using 392 microsatellite markers, HLA and CTLA-4 genes. We used the non-parametric sib-pair method in 123 Japanese sib-pairs affected with AITD to identify the possible susceptibility loci for AITD.

RESULTS

ASP linkage analysis

Whole genome linkage analysis using the ASP method with 392 microsatellite markers was carried out on 123 ASPs with AITD, including 67 GD-ASPs, 25 HT-ASPs and 31 GD-HT

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Table 1. Characteristics of families with AITD

	GD	HT	Mixed	Total
Families with two affected sibs	65	19	21	105
Families with three affected sibs	1	3	3	7
Families with five affected sibs	0	0	1	1
No. of families	66	22	25	113
No. of informative ASPs	67	25	31	123

mixed families' ASPs (Table 1). Multipoint linkage analysis on AITD-ASPs as a whole, GD-ASPs and HT-ASPs at all chromosomes using the MAPMAKER/SIBS program revealed 19 regions on 14 chromosomes (1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 15, 18 and 22) where the maximum LOD score (MLS) was >1, and nine other chromosomes (4, 7, 14, 16, 17, 19, 20, 21 and X) which did not have the region where the MLS was >1 (Table 2; Fig. 1).

The highest MLS for AITD was 3.14 at D5S436 on chromosome 5. The MLSs at D5S436 for GD, HT and GD-HT mixed ASPs were 1.48, 0.42 and 0.90, respectively. These findings suggest that D5S436 will be closely linked to a common susceptibility locus for GD and HT. Figure 2A and B show a multipoint LOD score map of AITD, GD and HT and the allele sharing proportion corrected by MAPMAKER/SIBS for AITD on chromosome 5, respectively. As shown in Table 2, besides D5S436, there were eight regions (D5S407, D6S462, D8S272, D11S905, D12S336, D13S159, D18S487 and D22S423) where the MLS was >1 in AITD-ASPs.

The highest MLS for HT was 3.77 at D8S272 on chromosome 8. The MLSs at D8S272 for AITD and GD were 2.31 and 1.13, respectively (Table 2). Figure 3A and B show a multipoint LOD score map of AITD, GD and HT, and the allele sharing proportion of HT-ASPs on chromosome 8, respectively. These results provide suggestive evidence for linkage of D8S272 specifically to HT, although a possible linkage of this marker with GD should not be ignored. On the other hand, in the case of GD-ASPs, none of the markers

Table 2. Summary of sib-pair analysis in AITD, GD and HT families

Chromosome	Marker	cM[a]	Het.[b]	Multipoint LOD score		
				All families	GD+GD families	HT+HT families
1	D1S213	232	0.83	0.628	**1.119**	0.301
2	D2S335	179	0.76	0.000	0.000	**1.165**
	D2S125	264	0.82	0.074	**1.181**	0.004
3	D3S1580	212	0.88	0.219	0.000	**1.635**
5	D5S407	67	0.86	**1.945**	0.614	**1.940**
	D5S436	156	0.79	**3.141**	**1.475**	0.422
6	D6S462	89	0.58	**1.007**	0.913	0.001
	D6S281	188	0.71	0.758	0.173	**1.869**
8	D8S272	149	0.79	**2.309**	**1.130**	**3.768**
9	D9S1677	108	0.79	0.546	0.188	**1.687**
10	D10S249	0	0.78	0.503	**1.106**	0.000
11	D11S905	53	0.60	**1.079**	0.576	0.570
12	D12S336	22	0.74	**1.452**	**1.049**	0.530
13	D13S159	74	0.80	**1.109**	0.185	0.874
15	D15S1007	22	0.85	0.653	**1.479**	0.022
	D15S130	100	0.73	0.604	**1.078**	0.168
18	D18S53	40	0.78	0.670	0.081	**1.112**
	D18S487	77	0.66	**1.118**	0.824	0.531
22	D22S423	42	0.77	**1.516**	**1.178**	0.046

LOD scores >1 are in bold.
[a]The distance of the marker from the p-terminal end of the chromosome in cM.
[b]Heterozygosities of the marker estimated for all individuals.

examined showed an MLS >2, and the highest MLS was 1.48 at chromosome 15 (D15S1007) (Table 2). The MLSs at this marker for AITD and HT were 0.65 and 0.02, respectively.

To examine the relation between the population we analyzed and the loci reported to be linked to these diseases, the markers, including HLA-DPB1, CTLA-4[AT]n, D18S487 (IDDM6) (15,16), D6S257 (AITD-1), D12S351 (HT-2), D13S173 (HT-1), D14S81 (GD-1), D20S195 (GD-2) and DXS8020 (GD-3) (9–14), were used to examine linkage with the diseases. The MLS for AITD at the marker D18S487 near IDDM6 was 1.12, supporting the susceptibility locus in a previous report (16); however, all other markers showed an MLS <1 (Table 3).

Association of HLA and CTLA-4 with AITD

Association analyses of HLA-DPB1*0501 and HLA-DRB4*0101 with AITD, which we previously reported to be associated with GD (7) and HT (8), respectively, are summarized in Table 4. A statistically significant increase in the frequency of HLA-DPB1*0501 was shown in 113 unrelated AITD patients [*P* value = 0.00003, odds ratio (OR) = 3.1] and 78 unrelated GD patients (*P* value = 0.00004, OR = 3.8). The significant increase in the frequency of HLA-DRB4*0101 was observed in AITD (*P* value = 0.00099, OR = 2.6), GD (*P* value = 0.0081, OR = 2.2), and HT patients (*P* value = 0.024, OR = 2.8). These results support data in our previous studies, and would validate the population analyzed in this study.

The frequency of the 106 bp allele of CTLA-4[AT]n in the present study was 36.0% in 111 AITD patients, 40.8% in 76 GD patients, 25.7% in 35 HT patients and 31.7% in 218 controls, which suggests a weak association of the 106 bp allele of CTLA-4[AT]n with GD (*P* = 0.041), but not with AITD and HT. The G allele and the genotype of GG of the A/G polymorphism in exon 1 of the CTLA-4 gene were also reported to be associated with GD (21,22,27), HT (23) and AITD (26). We found no significant association of AITD, GD and HT with the G allele and the genotype of GG (data not shown).

DISCUSSION

ASP analysis using 392 microsatellite markers in 123 Japanese AITD-ASPs showed an MLS >3 at 5q31–q33 on AITD-ASPs, which means that a susceptibility gene for AITD may exist around this region. A cytokine gene cluster, including IL-3, IL-4, IL-5, IL-9, IL-13 and a granulocyte-macrophage colony-stimulating factor were mapped within 5q31–q33, a region reported to be linked to allergic diseases such as asthma and atopic dermatitis (29–31). AITD is characterized by an abnormality in immune regulation, hence a disturbed cytokine secretion may be involved in the pathogenesis of AITD. Serum levels of IL-5 secreted from Th2 cells were found to be increased in thyrotoxic patients with GD (32). Thus the possible linkage of 5q31–q33 to AITD provides the basis for further investigation of these cytokine genes in AITD; however, it is also important to explore other unknown genes in this region.

The MLS at D8S272 for HT exceeded 3. The frequency of the pairs sharing two alleles in HT-ASPs was extremely high (Z2 = 0.724, Z1 = 0.189, Z0 = 0.087) at D8S272 (Fig. 2). It is interesting to note that the thyroglobulin gene is mapped close to D8S272 since HT is characterized by increased serum levels of the anti-thyroglobulin antibody. Polymorphisms of the thyroglobulin gene were noted in patients with congenital goiter and were suggested to cause defective thyroglobulin synthesis (33,34). Although there are pathologic differences between HT and congenital goiter, polymorphisms of the thyroglobulin gene might vary functions of thyroglobulin, contributing to the susceptibility to HT. As there seem to be several genes in the 8q23–q24 region apart from the thyroglobulin gene (35), we should examine not only the thyroglobulin gene but also other known and unknown genes in this candidate region.

Among the nine regions reported to be linked to AITD in Caucasians, only D18S487 showed an MLS >1 (MLS = 1.12). The MLSs at D18S487 for GD and HT were 0.82 and 0.53, respectively (Table 3). Therefore, D18S487 may be a common susceptibility locus for GD and HT. This region was found to be linked to other autoimmune diseases, such as type 1 diabetes (36–38), rheumatoid arthritis (39) and systemic lupus erythematosus (40), thereby suggesting that this region may

Table 3. Multipoint LOD scores in AITD, GD and HT families at loci reported to be linked to AITD

Locus	Marker name	Chromosomal location	All families (123 ASPs)	GD+GD families 66 ASPs)	HT+HT families (25 ASPs)
HLA	DPB1	6p21.3	0.000	0.000	0.000
CTLA-4	CTLA-4[AT]n	2q33	0.000	0.000	0.462
IDDM6	D18S487	18q21	**1.118**	0.824	0.531
AITD-1	D6S257	6p	0.542	0.034	0.062
GD-1	D14S81	14q31	0.000	0.000	0.005
GD-2	D20S195	20q11.2	0.000	0.000	0.146
GD-3	DXS8020	Xq21	0.007	0.000	0.726
HT-1	D13S173	13q32	0.303	0.099	0.577
HT-2	D12S351	12q22	0.263	0.604	0.000

LOD scores >1 are in bold.

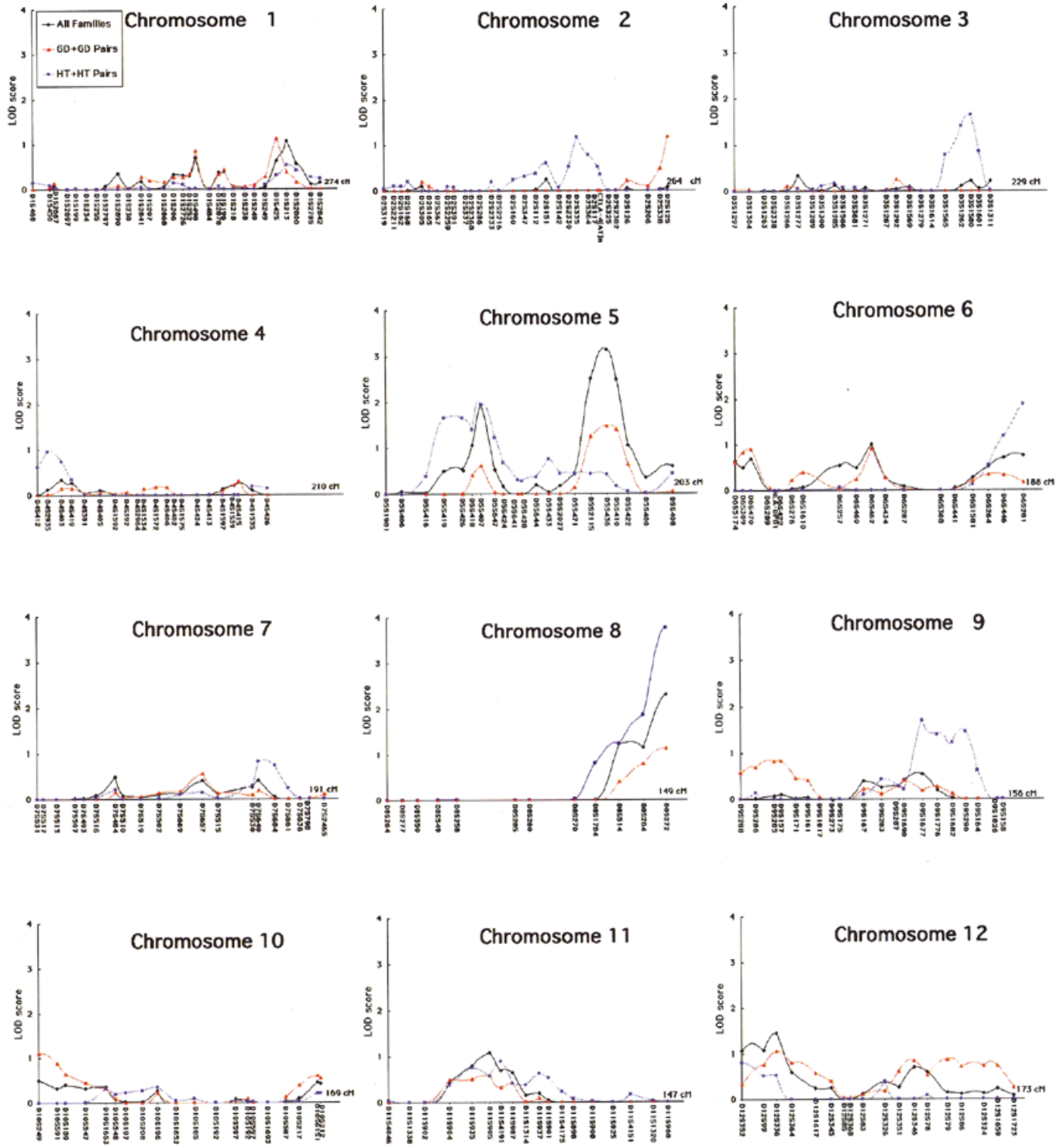
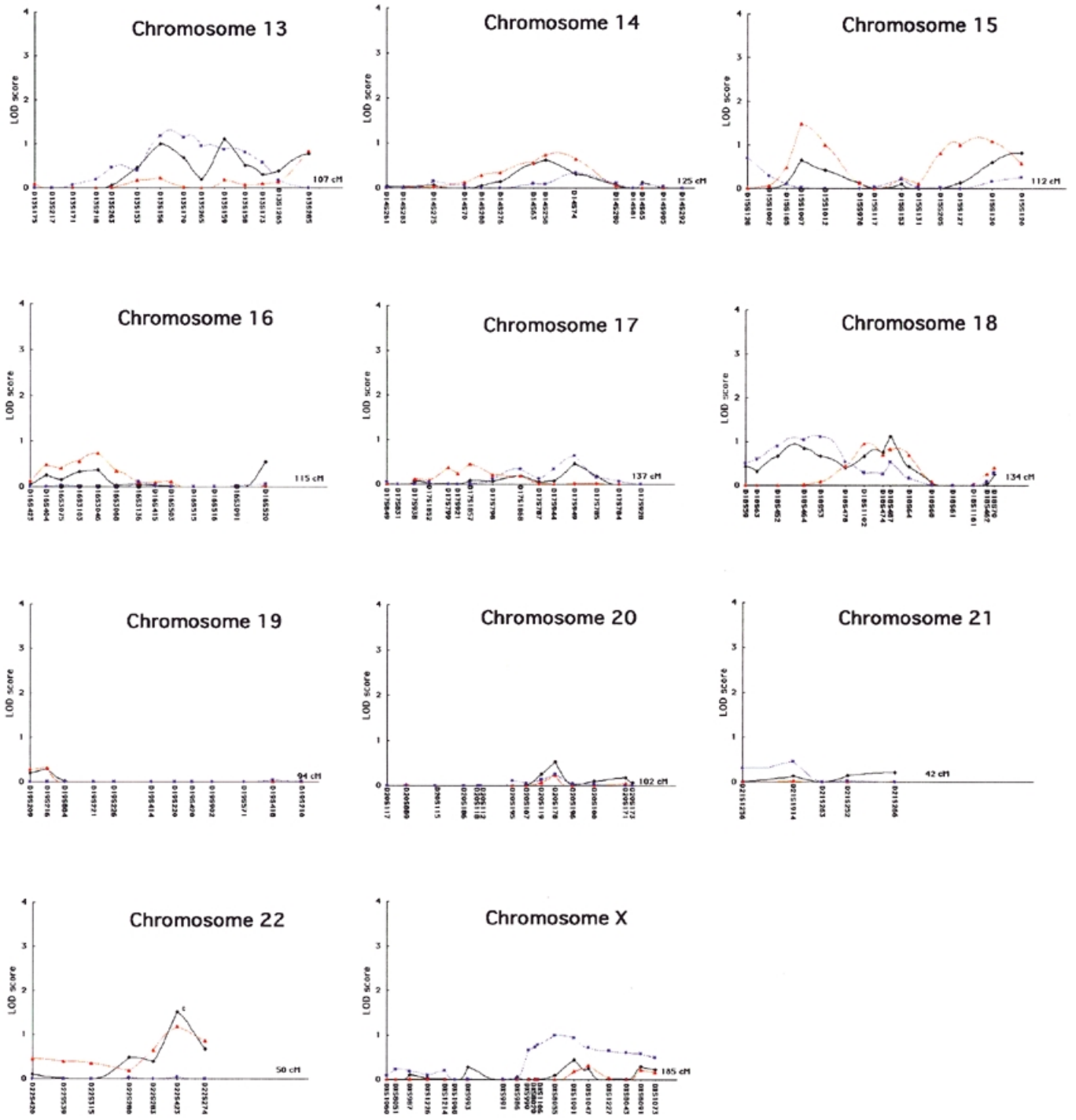


Figure 1. Multipoint LOD score map of AITD (black), GD (red) and HT (blue) by linkage analysis of 392 markers in 113 Japanese families.

include a common susceptibility gene for these autoimmune diseases.

Other suggested susceptibility loci for AITD, HLA (6p21.3), CTLA-4 (2q33) (15), GD-1 (14q31), GD-2 (20q11.2), GD-3 (Xq21), AITD-1 (6p), HT-1 (13q32) and HT-2 (12q22) (9–14) did not show an MLS >1. This discrepancy may be explained by the following reasons. (i) The ethnic difference between subjects used in our study and previous studies. (ii) The difference in ascertainment of the families analyzed. The analyses in previous whole genome studies were done in order to detect HT (or GD) susceptibility loci, based on the assumption that GD (or HT) patients were unaffected in GD-HT mixed



families. In our study, GD-HT mixed ASPs including both GD and HT were excluded, and analyses were made on HT-HT ASPs or GD-GD ASPs to detect HT- or GD-specific loci. (iii) A weak statistical power of the current study may also be responsible.

Our first attempt of the whole genome analysis of Japanese AITD demonstrated a suggestive linkage of 5q31–q33 to AITD and 8q23–q24 to HT. These data will need to be confirmed in larger numbers of patients and in additional ethnic populations.

MATERIAL AND METHODS

Families

A total of 236 Japanese members affected with AITD in 113 families (61 from Ito Hospital, 26 from Kuma Hospital and 26 from Kyoto University Hospital) were genotyped. Each participant was interviewed and examined and gave written informed consent before participating in this study. There were

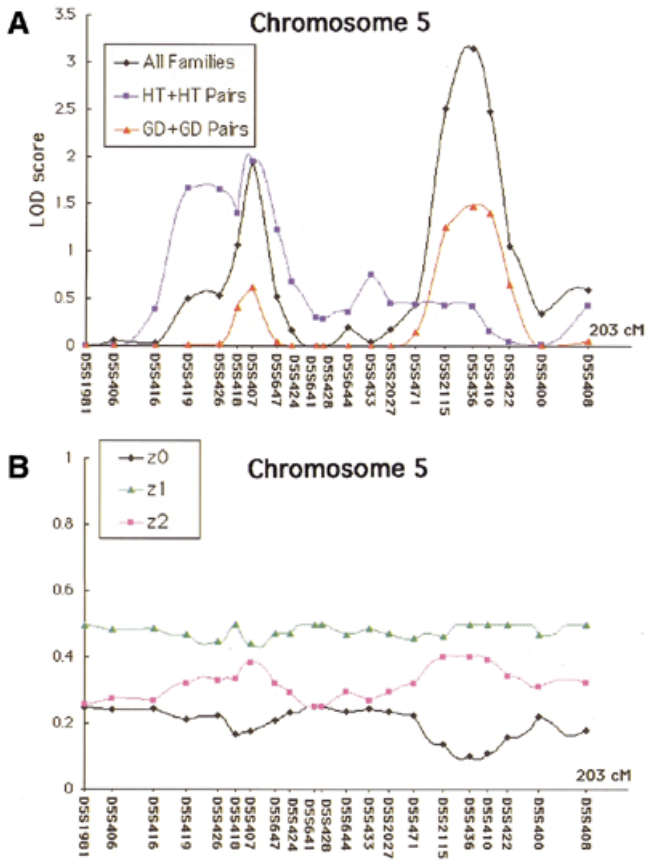


Figure 2. (**A**) Multipoint LOD score map of AITD (black), GD (red) and HT (blue) on chromosome 5. (**B**) Allele sharing proportion in AITD families generated by MAPMAKER/SIBS program on chromosome 5. Z0 (black), Z1 (green) and Z2 (pink), mean sharing 0, 1, and 2 alleles identical by descent (IBD), respectively.

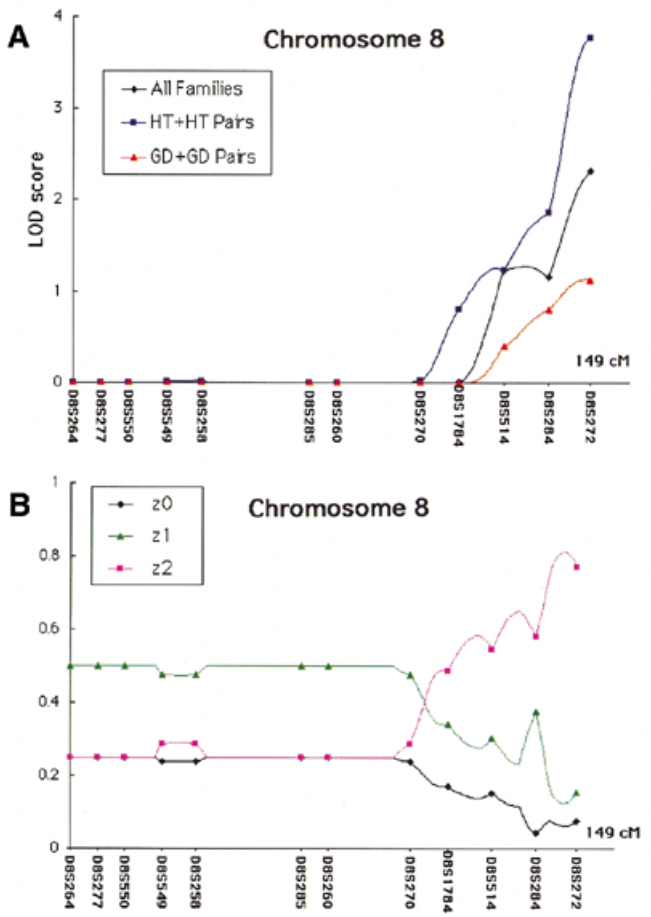


Figure 3. (**A**) Multipoint LOD score map of AITD (black), GD (red) and HT (blue) on chromosome 8. (**B**) Allele sharing proportion in HT families generated by MAPMAKER/SIBS program on chromosome 8. Z0 (black), Z1 (green) and Z2 (pink), mean sharing 0, 1, and 2 alleles IBD, respectively.

67 GD-ASPs, 25 HT-ASPs and 31 GD-HT mixed ASPs (Table 1). Of the 236 affected individuals, 200 were women and 36 were men. Diagnosis of thyroid disease was established based on clinical findings and results of routine examinations; circulating thyroid hormone and thyroid stimulating hormone (TSH) concentrations, serum levels of antibodies against thyroglobulin, thyroid microsomes and TSH receptors, ultrasonography, $^{[99m]}TCO_4^-$ (or $[^{123}I]$) uptake and thyroid scintigraphy.

Table 4. Association between HLA and AITD

	AITD *n* = 113 (%)	*P* value	OR (95% CI)	GD *n* =78 (%)	*P* value	OR (95% CI)	HT *n* = 35 (%)	*P* value	OR (95% CI)	Control[a] *n* = 317 (%)	GD in past study[a] *n* =76 (%)	*P* value	OR (95% CI)
DPB1*0501 (+)	94 (83.2)	0.00003	3.1 (1.8–5.3)	67 (85.9)	0.00004	3.8 (1.9–7.5)	27 (77.1)	NS	2.1 (ND)	195 (61.5)	68 (89.5)	<0.00001	5.3 (2.5–11.4)
DPB1*0501 (–)	19 (16.8)			11 (14.1)			8 (22.9)			122 (38.5)	8 (10.5)		
	AITD *n* = 113 (%)	*P* value	OR (95% CI)	GD *n* = 78 (%)	*P* value	OR (95% CI)	HT *n* = 35 (%)	*P* value	OR (95% CI)	Control[b] *n* = 317 (%)	HT in past study[b] *n* = 71 (%)	*P* value	OR (95% CI)
DRB4*0101 (+)	91 (80.5)	0.00099	2.6 (1.4–4.0)	62 (79.5)	0.00807	2.2 (1.2–4.0)	29 (82.9)	0.02371	2.8 (1.1–6.8)	202 (63.7)	63 (88.7)	0.00004	4.5 (2.1–9.7)
DRB4*0101 (–)	22 (19.5)			16 (20.5)			6 (17.1)			115 (36.3)	8 (11.3)		

n, number of patients; CI, confidence interval; NS, not significant; ND, not determined.
[a]Dong *et al.* (7).
[b]Wan *et al.* (8).

Genotyping

DNA was isolated from peripheral blood cells using QIAamp DNA Blood Midi Kits (Qiagen). In addition to ABI PRISM Linkage Mapping Set Version 2, we genotyped microsatellite markers around loci reported to be linked to AITD, CTLA-4[AT]n (20), D14S81, D18S487, D20S118 and DXS8020 given on the Genethon genetic linkage map. PCR was performed using the GeneAmp PCR System 9700 with standard protocols. Genotyping of 392 microsatellite markers, ~9.0 cM apart, was performed using an ABI 377 automatic sequencer (Applied Biosystems). Analyses and assignment of the marker alleles were done with GENESCAN and GENOTYPER (ABI) software.

HLA class II were genotyped using a PCR-sequence-specific-oligonucleotide probe (SSOP) method. Genomic DNA was amplified for the second exon of the *DPB1* gene and the second exon of the *DRB* gene. PCR and hybridization procedures with SSOPs have been described previously (7,8).

The CTLA-4A/G polymorphism in exon 1 of the CTLA-4 gene was amplified using primers 5′-CCA CGG CTT CCT TTC TCG TA-3′ and 5′-AGT CTC ACT CAC CTT TGC AG-3′, and the restriction fragment length polymorphism analysis was performed on 4 µl of PCR products, digested with 2.5 U of *Bst*71I (Promega) in a final volume of 20 µl under appropriate buffer conditions at 50°C for 3 h (15,24). DNA fragments were resolved in 1.5% agarose gels stained with ethidium bromide.

Linkage and association analysis

Multipoint analysis for LOD score was carried out on weighted all pairs, using the MAPMAKER/SIBS program (41). Allele frequencies were estimated using data from unrelated individuals.

Association analyses between controls and patients were carried out using a contingency 2×2 table to calculate an OR and χ^2. 113 AITD probands were analyzed, including 78 GD and 35 HT probands. As a control population, 317 healthy unrelated Japanese persons (7,8) were studied in the HLA association analysis, and 279 unrelated controls were studied in the CTLA-4 association analysis.

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