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SHORT COMMUNICATION

# HLA and CTLA4 polymorphisms may confer a synergistic risk in the susceptibility to Graves' disease

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**Graves' disease (GD) is an autoimmune disease characterized by hyperthyroidism due to the presence of autoantibodies against thyroid-stimulating hormone receptor, which is measured as thyroid-stimulating hormone-binding inhibitory immunoglobulin (TBII). Most of the GD patients are TBII-positive, but TBII is undetectable in a proportion of GD patients. We previously reported the association of HLA-A*02 and -DPB1*0501 with TBII-positive GD, whereas TBII-negative GD showed association with HLA-A*02 and DPB1*0202. Recently, polymorphisms of cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) gene are reported to be associated with GD. In this study, we investigated 329 (240 TBII-positive and 89 TBII-negative) GD patients and 378 controls for the polymorphisms in HLA-A, -DPB1 and CTLA4 (CT60, rs3087243, A/G) to investigate the contribution of these factors in the susceptibility to GD. A significant association with CTLA4 was found for the TBII-positive GD (G carriers in patients vs controls, 97.1 vs 91.8%; odds ratio (OR)=2.97, 95% confidence interval=1.29–6.87, *P*=0.008), but the association was weak and not significant for the TBII-negative GD (94.4 vs 91.8%; OR=1.50, 95% confidence interval=0.57–3.98, *P*=0.41). Stratification analyses suggested a possible synergistic interaction of CTLA4 with HLA-A*02 and -DPB1*0501 in the susceptibility to TBII-positive GD.**
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## INTRODUCTION

Graves' disease (GD) is a typical organ-specific autoimmune disease affecting thyroid gland, in which autoantibodies against the receptors for thyroid-stimulating hormone induce hyperthyroidism. The autoantibodies has been measured as thyroid-stimulating antibodies (TSAb) by a biological assay or as thyroid-stimulating hormone-binding inhibitory immunoglobulins (TBII) by a solid phase assay.[1] Although most of the GD patients are positive for both TBII and TSAb, a proportion of the patients carry low or undetectable level of TBII (TBII-negative GD).[2] However, TSAb can be detected in the TBII-negative GD, and it was reported that the TBII-negative patients exhibited mild goiter and followed benign prognosis, implying that the TBII-negative GD might be a clinical subtype different from the ordinary TBII-positive GD.[3]

Etiological mechanisms of GD are not fully understood, but certain genetic factors should be involved in the pathogenesis because of familial aggregation of the disease.[4] To decipher the genetic factors candidate gene approaches have been used, and it is well documented that human leukocyte antigen (HLA) polymorphisms are associated with GD, such that HLA-B*08-DRB1*03-DQA1*05-DQB1*02 haplotype showed strong association in European populations.[5,6] However, the distribution of HLA alleles and haplotypes are quite different in different ethnic groups, and the European GD-associated HLA haplotype is virtually absent in Japanese.[7] We previously reported that HLA-A*02 and DPB1*0501 are associated with Japanese GD.[8] In addition, we demonstrated that HLA-A*02 and DPB1*0202 showed association with TBII-negative GD, indicating that the TBII-negative GD was different in HLA-linked genetic factors from the TBII-positive GD.[9]

There are several other genetic factors associated with GD, including polymorphisms in the gene for cytotoxic T-lymphocyte-associated antigen-4 (*CTLA4*).[10] *CTLA4* encodes a co-stimulatory molecule of T cells, which is involved in the regulation of T-cell activation,[11,12] and it has been reported that the polymorphisms in exon 1 and 3′-UTR region are associated with GD as well as Hashimoto thyroiditis, another autoimmune thyroid disease, in European and Asian populations.[10,13–15] However, it is not clear how the CTLA4 and HLA polymorphisms would confer the risk to GD; that is, they operate independently or synergistically in determining the genetic risk. To clarify the issue, we analyzed a CTLA4 polymorphism (rs3087243, also called as CT60) in addition to HLA-A*02, DPB1*0202 and DPB1*0501 in association with GD.

## MATERIALS AND METHODS

### Subjects

A total of 329 Japanese GD patients (240 TBII-positives and 89 TBII-negatives) and 378 randomly selected Japanese healthy controls were the subjects. The

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TBII-negative patients were randomly selected from the previously reported 97 patients,[9] whereas the TBII-positive patients were those analyzed previously[8] ($n$=142) and newly recruited patients ($n$=98). The diagnosis of GD and assay for TBII were performed as described previously.[2,3,9] Blood sample to prepare genomic DNA was obtained from each subject under a given informed consent. The study protocol was approved by the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University.

### Genotyping

Detection of HLA-A*02 and HLA-DPB1 genotyping was carried out as described previously.[9] The CTLA4 polymorphism (CT60, rs3087243) was analyzed by the PCR-restriction-fragment length polymorphism method using HpyCh4IV.[10,16]

### Statistical analysis

Frequencies of alleles and genotypes were compared between the patients and controls using $\chi^2$-test. Strength of the association was expressed by odds ratio (OR). A stratification analysis was carried as described by Svejgaard and Ryder[17] to test the independency or synergistic effects of two different alleles. $P$-values were corrected for multiple testing by the number of tested markers, HLA-A*02, DPB1*0501, DPB1*0202 and CTLA4-CT60-G ($n$=4). The association was considered to be significant when the corrected $P$-value was less than 0.05.

## RESULTS AND DISCUSSION

Table 1 shows the carrier frequencies of HLA-A*02, -DPB1*0501 and -DPB1*0202 in the studied populations, demonstrating that the frequencies of disease-associated HLA carriers were significantly high in the patients. When the GD patients were divided into TBII-positive and -negative groups, HLA-A*02 and DPB1*0501 were associated with TBII-positive GD, although the association of HLA-A*02 was weak and marginal because it lost the significance after the Bonferroni's correction for multiple testing. In contrast, HLA-A*02 and DPB1*0202 were significantly associated with TBII-negative GD.

The analysis of a CTLA4 polymorphism CT60 demonstrated that the association with G allele was strong and significant for TBII-positive GD (OR=2.97, 95% confidence interval=1.29–6.87, $P$=0.031), but it was weak and not significant in TBII-negative GD (OR=1.50, 95% confidence interval=0.57–3.98, $P$=nonsignificant) (Table 1). Because TSAb can be detected in almost all of TBII-positive GD patients, while only a part of TBII-negative GD patients were positive for TSAb, the observation that the association with CT60-G allele was weak and not significant for the TBII-negative GD might be due to that CT60-G allele was associated with the presence of TSAb. Among the TBII-negative GD patients, 31 (34.8%) were TSAb-positive, whereas TSAb was not detected in the other 58 patients. Frequency of CTLA4-CT60-G carrier was 93.5% in the TSAb-positive patients, whereas it was 94.8% in the TSAb-negative patients. These findings suggested that the CTLA4-CT60-G allele was not associated with the presence of TSAb. We previously reported that the absence of TBII might be a predictor of good prognosis, because the efficacy of medication and the disease-associated HLA-DPB1 allele was different from the typical GD positive for TBII.[8] In this study, we also demonstrated that the association with CT60 was weak for TBII-negative GD, implying that the contribution of CTLA4 in the pathogenesis was relatively small in the TBII-negative GD, further supporting that the absence of TBII was a good predictor for prognosis of GD.

We next performed a stratification analysis of three different GD-associated alleles, HLA-A*02, DPB1*0501 and CTLA4-CT60-G, in the TBII-positive GD (Table 2). It was found that the ORs of HLA-DPB1*0501 were 3.28 and 3.30 in the presence and absence of CTLA4-CT60-G, respectively, suggesting that CTLA4-linked genetic factor would increase the HLA-DPB1-linked risk (Table 2b-1, test {3} and {4}). Similarly, ORs of CTLA4-CT60-G were 3.03 and 3.05 in the presence and absence of HLA-DPB1*0501, respectively, showing that HLA-DPB1-linked genetic factor would increase the CTLA4-linked risk (Table 2b-1, test {5} and {6}). In addition, the presence of both HLA-DPB1*0501 and CTLA4-CT60-G conferred OR of 9.99 (Table 2b-1, test {8}), which was much higher than HLA-DPB1*0501 or CTLA4-CT60-G alone (OR=−3.30 or 3.05, Table 2b-1, test {4} or {6}, respectively), although it was not significant because the 95% confidence interval of ORs for each category showed a considerable overlapping as shown in Table 2b-1. These observations suggested a possible synergistic role of HLA-DPB1*0501 and CTLA4-CT60-G in the susceptibility to TBII-positive GD. It was also found that HLA-A*02 and CTLA4-CT60-G conferred a possible synergistic risk, albeit to a less extent, because the presence of both HLA-A*02 and CTLA4-CT60-G conferred OR of 3.75 (Table 2b-2, test {8}), which was much higher than HLA-A*02 or CTLA4-CT60-G alone (OR=1.04 or 2.50, Table 2b-2, test {4} or {6}, respectively), although it was not statistically significant because of the overlapping of 95% confidence interval of ORs (Table 2b-2). Because there is no linkage disequilibrium between HLA-A*02 and DPB1*0501,[7] the findings in this study implied a synergistic contribution of HLA-linked factors and CTLA4-linked factor in the susceptibility.

**Table 1 Frequencies of HLA-A*02, -DPB1*0501, -DPB1*0202 and CTLA4-CT60-G in the Japanese populations**

| *Genetic marker* | *GD (n=329)* | *TBII-positive GD (n=240)* | *TBII-negative GD (n=89)* | *Control (n=378)* |
|---|---|---|---|---|
| HLA-A*02 | 54.4 %, OR=1.68, 95% CI=1.25–2.26, $P$=0.0025 | 51.3%, OR=1.48, 95% CI=1.07–2.05, $P$=NS ($P$=0.018) | 62.9%, OR=2.39, 95% CI=1.48–3.85, $P$=0.0011 | 41.5 % |
| HLA-DPB1*0501 | 81.2%, OR=2.56, 95% CI=1.81–3.62, $P$=0.00000025 | 84.6%, OR=3.26, 95% CI=2.17–4.91, $P$=0.000000019 | 71.9%, OR=1.52, 95% CI=0.92–2.53, $P$=NS | 62.7 % |
| HLA-DPB1*0202 | 12.8%, OR=1.90, 95% CI=1.14–3.16, $P$=0.048 | 10.8%, OR=1.58, 95% CI=0.90–2.78, $P$=NS | 18.0%, OR=2.85, 95% CI=−1.46–5.56, $P$=0.006 | 7.1 % |
| CTLA4-CT60-G | 96.4%, OR=2.36, 95% CI=1.19–4.67, $P$=0.046 | 97.1%, OR=2.97, 95% CI=1.29–6.87, $P$=0.031 | 94.4%, OR=1.50, 95% CI=0.57–3.98, $P$=NS | 91.8 % |

Abbreviations: CI, confidence interval; GD, Graves' disease; NS, nonsignificant (>0.05); OR, odds ratio; TBII, thyroid-stimulating hormone-binding inhibitory immunoglobulin.

**Table 2 Stratification analysis of HLA and CTLA4 in GD**

| *HLA-A*02* | *HLA-DPB1*0501* | *CTLA4-CT60-G* | *TBII-positive GD* (n=*240*) | *Control* (n=*378*) |
|---|---|---|---|---|
| *(a) Basic data* | | | | |
| Presence (+) | Presence (+) | | 109 (45.4) | 98 (25.9%) |
| Presence (+) | Absence (−) | | 14 (5.8%) | 59 (15.6%) |
| Absence (−) | Presence (+) | | 94 (39.2%) | 139 (36.8%) |
| Absence (−) | Absence (−) | | 23 (9.6%) | 82 (21.7%) |
| Presence (+) | | Presence (+) | 120 (50.0%) | 144 (38.1%) |
| Presence (+) | | Absence (−) | 3 (1.3%) | 13 (3.4%) |
| Absence (−) | | Presence (+) | 113 (47.1%) | 203 (53.7%) |
| Absence (−) | | Absence (−) | 4 (1.7%) | 18 (4.8%) |
| | Presence (+) | Presence (+) | 197 (82.1%) | 217 (57.4%) |
| | Presence (+) | Absence (−) | 6 (2.5%) | 20 (5.3%) |
| | Absence (−) | Presence (+) | 36 (15.0%) | 130 (34.4%) |
| | Absence (−) | Absence (−) | 1 (0.4%) | 11 (2.9%) |

*(b) Stratification analysis in TBII-positive GD*

| | *Comparison* | | *Individual association* | | *Individual association for factor A* | | *Individual association for factor B* | | *Difference between A and B* | *Combinatory association* |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Factor A* | *Factor B* | *Test {1}* | *Test {2}* | *Test {3}* | *Test {4}* | *Test {5}* | *Test {6}* | *Test {7}* | *Test {8}* |
| | *DPB1*0501* | *CTLA4- CT60-G* | $OR_A$ | $OR_B$ | *++ vs −+* | *+− vs −−* | *++ vs +−* | *−+ vs −−* | *+− vs −+* | *++ vs −−* |
| | | | | | $OR_{A,non-B}$ | | $OR_{non-A,B}$ | | | $OR_{A,B}$ |
| *(1) HLA-DPB1*0501 and CTLA4- CT60-G* | | | | | | | | | | |
| OR | | | 3.26 | 2.97 | 3.28 | 3.30 | 3.03 | 3.05 | 1.08 | 9.99 |
| 95% CI | | | 2.17–4.91 | 1.29–6.87 | 2.16–4.97 | 0.35–31.04 | 1.19–7.69 | 0.38–24.39 | 0.40–2.90 | 1.28–78.06 |
| *P*-value | | | 4.8E-9 | 0.0077 | 8.9E-9 | NS | 0.015 | NS | NS | 0.0072 |

| | *Comparison* | | *Individual association* | | *Individual association for factor A* | | *Individual association for factor B* | | *Difference between A and B* | *Combinatory association* |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Factor A* | *Factor B* | *Test {1}* | *Test {2}* | *Test {3}* | *Test {4}* | *Test {5}* | *Test {6}* | *Test {7}* | *Test {8}* |
| | *HLA-A*02* | *CTLA4- CT60-G* | $OR_A$ | $OR_B$ | *++ vs −+* | *+− vs −−* | *++ vs +−* | *−+ vs −−* | *+− vs −+* | *++ vs −−* |
| | | | | | $OR_{A,non-B}$ | | $OR_{non-A,B}$ | | | $OR_{A,B}$ |
| *(2) HLA-A*02 and CTLA4- CT60-G* | | | | | | | | | | |
| OR | | | 1.48 | 2.97 | 1.50 | 1.04 | 3.61 | 2.50 | 0.41 | 3.75 |
| 95% CI | | | 1.07–2.05 | 1.29–6.87 | 1.07–2.09 | 0.20–5.45 | 1.01–12.97 | 0.83–7.58 | 0.12–1.49 | 1.24–11.38 |
| *P*-value | | | 0.018 | 0.0076 | 0.018 | NS | 0.037 | NS | NS | 0.013 |

Abbreviations: CI, confidence interval; GD, Graves' disease; NS, nonsignificant (>0.05); OR, odds ratio; TBII, thyroid-stimulating hormone-binding inhibitory immunoglobulin.

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