A C/T Polymorphism in the 5’ Untranslated Region of the CD40 Gene Is Associated with Later Onset of Graves’ Disease in Japanese

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Abstract. Graves’ disease (GD) is an autoimmune disorder with genetic predisposition. CD40, which stimulates lymphocyte proliferation and differentiation, is an important immunomodulator and is expressed in the thyroid follicular cells as well as antigen-presenting cells. A single nucleotide polymorphism (SNP) at position –1 of the Kozak sequence of the CD40 gene has been reported to be associated with the development of GD. The aim of the present study was to investigate whether CD40 gene polymorphism confers susceptibility to GD in Japanese. CD40 gene polymorphisms were studied in Japanese GD patients (n = 324) and healthy control subjects without anti-thyroid autoantibodies or a family history of autoimmune disorders (n = 229). A C/T polymorphism at position –1 of the CD40 gene was measured using the polymerase chain reaction restriction fragment length polymorphism. There was no significant difference in allele or genotype frequency of the CD40 SNP between GD and control subjects. There was a significant decrease in the TT genotype frequency in the GD patients, who developed GD after 40 years old, than those under 40 year of age. These data suggest that the SNP of CD40 gene is associated with susceptibility to later onset of GD in Japanese.

Key words: Polymorphism, CD40, Graves’ disease

GRAVES’ disease (GD) is an autoimmune disorder, characterized by the presence of anti-thyroid-stimulating hormone (TSH) receptor antibodies [1]. Several lines of research support the involvement of environmental factors, such as smoking, and genetic factors in both GD and Graves’ ophthalmopathy (GO) [2, 3]. The genetic susceptibility of these diseases is thought to be polygenic. It has been reported that major histocompatibility complex (MHC) gene [4, 5], cytotoxic T lymphocyte antigen-4 (CTLA-4) gene [6–8], interferon-γ (IFN-γ) gene [9, 10] and tumor necrosis factor-α (TNF-α) gene [11] polymorphisms are associated with GD and GO. However, none of these associations have been fully confirmed.

Recently, the susceptibility locus for GD has been mapped to chromosome 20q11 (GD-2) [12–14]. Furthermore, the association of the C/T polymorphism in the 5’-untranslated region of CD40 gene with GD has been reported in Caucasian [15] and Koreans [16]. However, the association could not be confirmed in United Kingdom (UK) Caucasians [17, 18].

CD40 is a member of the tumor necrosis factor receptor family [19], and expressed on antigen-presenting cells such as B cells, dendritic cells, thymic epithelial cells [20], and thyrocytes [21, 22]. The interaction of CD40 with its ligand induces T helper (Th) 2 immune response [23], resulting in driving thyroid autoimmunity in the direction of GD, and could influence the production of anti-TSH receptor antibodies in GD and clinical manifestation of GD. The blocking of CD40/CD40L interactions in murine models suppresses thyroiditis in these animals [24]. Therefore, the SNP of
the CD40 gene is a candidate for GD susceptibility.

The aim of the present study was to investigate whether CD40 gene polymorphism is associated with the development of GD and its clinical features.

Materials and Methods

Subjects

In total, 324 GD patients (73 males, 251 females; aged 11–83 years, mean ± SD age, 42.1 ± 16.1 years) being treated at Kurume University Hospital were enrolled in this study. GD diagnosis was determined by the presence of hyperthyroidism and serum anti-thyrotropin receptor antibodies (thyrotropin binding inhibiting immunoglobulin (TRAb) and thyroid stimulating autoantibodies) and/or an increased $^{123}$I uptake ratio with diffuse uptake. Ophthalmopathy was classified according to the system recommended by the American Thyroid Association (ATA) Committee [25]. One-hundred and two GD patients, 24 males and 78 females, showed ophthalmopathy defined as ATA class III or greater and were classified as GO. Two-hundred and twenty-two patients showed no ophthalmopathy (ATA class 0), signs of ophthalmopathy without symptoms (ATA class I), or only soft tissue involvement (ATA class II). One-hundred and seventy-six patients developed GD under 40 years old, and 148 patients did over 40 years old. Two hundred and twenty-nine healthy unrelated Japanese medical students and staff members (102 males and 127 females; aged 18–79 years, mean ± SD age, 30.1 ± 9.5 years) with no family history of autoimmune diseases and no detectable anti-thyroid autoantibodies were enrolled as control subjects. The study plan was reviewed and approved by the institutional review committee, and informed consent was obtained from all patients and control subjects.

CD40 gene polymorphism

Genomic DNA extracted from peripheral blood was subjected to polymerase chain reaction (PCR) to amplify the polymorphic regions. The 5'-untranslated region of the CD40 gene was amplified by PCR using CD40 primers originally reported by Tomer et al. [14]. PCR was performed using 50 ng genomic DNA, 0.5 U Taq DNA polymerase (Ampli Taq Gold®, Applied Biosystems, Foster City, CA), 0.5 μM of each primer (forward, 5'-CCTCTTCCCCAGAAGTCTTCC-3'; reverse, 5'-GAAACTCCTGGCGGTTGAAT-3') and 200 μM of each dNTP, 1.5 mM of MgCl$_2$ under the following conditions: 35 cycles of PCR consisting of denaturing for 30 sec at 95°C, annealing for 30 sec at 55°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C in a thermocycler (Gene Amp PCR system 9600, Perkin Elmer Applied Biosystems, Foster City, CA).

The PCR products were digested by 0.1 U of Sty I (Promega Corp., Madison, WI) at 37°C for 2.5 hours. $^{31}$I digests the PCR fragment 99 bp from the 3'-end, which serves as a control for assessing whether digestion is complete. It also digests 129 bp from the 5'-end of the fragment when the C nucleotide is present producing a 74 bp fragment. The digested PCR products were electrophoresed on 3% agarose gels to separate the fragments. Some of the PCR products were directly sequenced using an ABI sequencer (ABI PRISM™ 3100 Genetic Analyzer, Perkin Elmer Applied Biosystems) to determine the C/T polymorphism at position –1.

Laboratory test

Serum concentrations of free T3, free T4 and TSH were determined by enzyme immunoassays (EIAs). TRAb was measured by radioreceptor assay with a commercial kit (Dia Sorin Inc., Stillwater, MN), and anti-thyroglobulin (TgAb) and anti-thyroid peroxidase antibodies (TPOAb) were measured by radioimmunoassay using commercial kits (RSR Ltd., Cardiff, UK). The cut-off values for TRAb, TgAb and TPOAb were 10%, 0.3 kU/L and 0.3 kU/L, respectively.

Statistical analysis

The laboratory data were expressed as the means ± standard deviation (SD). Differences in the clinical data between groups were evaluated using a Student’s t test or Welch’s t test. The statistical significance of any differences in frequency between each polymorphic allele and genotype of the patient and control groups was evaluated using the $\chi^2$ test or Fisher’s exact probability test. In this study, a P-value of <0.05 was considered statistically significant.
Results

Association between the CD40 gene polymorphism and Graves’ disease

The distribution of alleles for both groups (control and GD) is in good agreement with the Hardy-Weinberg equilibrium. There was no significant difference in allele or genotype frequency of the CD40 gene polymorphism between GD and control subjects (Table 1).

Association between the CD40 gene polymorphism and ophthalmopathy

There was no significant difference in genotype or allele frequency of the CD40 gene polymorphism between the patients with evident ophthalmopathy (ATA class III or more; GO) and those without or with mild ophthalmopathy (ATA class 0–II; Table 1).

Association of the CD40 gene polymorphism with the age of the onset of Graves’ disease

There were two peaks in the age of onset of GD (Fig. 1). The TT genotype frequency was significantly smaller in GD patients who developed GD after 40 years old than those who developed before 40 years old \((\chi^2 = 6.975, \, P = 0.0306)\) and control subjects \((CC + CT \, vs. \, TT, \, \chi^2 = 4.290, \, P = 0.0383, \, Table \, 2)\). The association of the polymorphism of CD40 gene with GD was also observed, when the female GD patients \((n = 251)\) were analyzed (the TT genotype frequency; 8% in GD patients who developed after 40 years old vs. 17% in GD patients who developed before 40 years old, \(\chi^2 = 4.835, \, P = 0.0279, \, Table \, 3)\).

Association between the CD40 gene polymorphism and the severity of Graves’ hyperthyroidism

There were no significant differences in the levels of serum FT\(_4\), FT\(_3\), TRAb or TSAb among the genotypes of the CD40 gene polymorphism (Table 4).

Discussion

Graves’ disease is an organ-specific autoimmune disorder characterized by a diffuse goiter and thyroid hormone oversecretion as a result of thyrotropin recep-

![Fig. 1. Distribution of age at onset of Graves’ disease](image)

| Table 1. Relationships of CD40 gene polymorphisms with Graves’ disease and ophthalmopathy |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Genotype frequencies            | Control subjects N = 229         | Graves’ disease Total N = 324    | Ophthalmopathy                  |
|                                 | CC                              | 80 (35)                         | 121 (37)                        |
|                                 | CT                              | 108 (47)                        | 152 (47)                        |
|                                 | TT                              | 41 (18)                         | 51 (16)                         |
|                                 |                                  | \(\chi^2 = 0.594\) P = 0.7431    | \(\chi^2 = 1.183\) P = 0.5536    |
| Allele frequencies              | C                               | 268 (59)                        | 394 (61)                        |
|                                 | T                               | 190 (41)                        | 254 (39)                        |
|                                 |                                  | \(\chi^2 = 0.059\) P = 0.8018    | \(\chi^2 = 1.067\) P = 0.3015    |

Values in parentheses are percentages of the group. P values were calculated with \(\chi^2\) test. ATA: American Thyroid Association
tor antibody stimulation. Although the etiology of GD remains unclear, it is believed to be caused by a complex interaction between genetic and environmental factors. Recent genome-wide researches have provided evidence for the linkage of GD to loci on multiple chromosomes, including loci on chromosomes 20q, designated GD-2, which have been linked to GD in Caucasian populations [12–14].

A C/T polymorphism at position –1 of the CD40 gene, which is in the Kozak sequence of CD40 gene, may control the initiation of translation of the CD40 protein [15, 26]. Furthermore, CD40 expression has been demonstrated in thyroid [21, 22] and orbital tissues from GD patients [27], suggesting that CD40

| Table 2. Association between the CD40 gene polymorphism in patients with Graves’ disease and age at onset |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Age at onset of Graves’ disease | Control subjects | GD ≥40 yr vs. GD <40 yr | GD ≥40 yr vs. Control |
|                                 | N = 176 | N = 148 | N = 229 | N = 179 |
| Genotype frequencies | | | | |
| CC | 65 (37) | 56 (38) | 80 (35) | 70 (37) |
| CT | 75 (43) | 77 (52) | 108 (47) | 100 (53) |
| TT | 36 (20) | 15 (10) | 41 (18) | 29 (15) |
| CC + CT | 140 (80) | 133 (90) | 188 (82) | 171 (91) |
| TT | 36 (20) | 15 (10) | 41 (18) | 29 (15) |
| Allele frequencies | | | | |
| C | 205 (58) | 189 (64) | 268 (59) | 251 (59) |
| T | 147 (42) | 107 (36) | 190 (41) | 150 (40) |
| Values in parentheses are percentages of the group. P values were calculated with $\chi^2$ test. GD: Graves’ disease |

| Table 3. Association between the CD40 gene polymorphism in female patients with Graves’ disease and age at onset |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Age at onset of Graves’ disease | Control subjects | GD ≥40 yr vs. GD <40 yr | GD ≥40 yr vs. Control |
|                                 | N = 138 | N = 113 | N = 127 | N = 116 |
| Genotype frequencies | | | | |
| CC | 53 (39) | 41 (36) | 42 (33) | 39 (34) |
| CT | 61 (44) | 63 (56) | 62 (49) | 61 (49) |
| TT | 24 (17) | 9 (8) | 23 (18) | 16 (14) |
| CC + CT | 114 (83) | 104 (92) | 104 (82) | 100 (87) |
| TT | 24 (17) | 9 (8) | 23 (18) | 20 (17) |
| Allele frequencies | | | | |
| C | 167 (61) | 145 (64) | 146 (57) | 137 (59) |
| T | 109 (39) | 81 (36) | 108 (43) | 99 (40) |
| Values in parentheses are percentages of the group. P values were calculated with $\chi^2$ test. GD: Graves’ disease |

| Table 4. Association of CD40 gene polymorphisms with laboratory features of patients with Graves’ disease |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| genotype | No | FT$_3$ (pg/ml) | FT$_4$ (ng/dl) | TRAb (%) | TSAb (%) |
| CC | 105 | 7.30 ± 7.20 | 2.78 ± 2.76 | 30.6 ± 60.9 | 400 ± 632 |
| CT | 138 | 7.44 ± 5.46 | 3.02 ± 2.73 | 26.1 ± 27.2 | 395 ± 500 |
| TT | 48 | 6.81 ± 4.79 | 2.81 ± 2.49 | 30.3 ± 30.0 | 421 ± 435 |
| No, number of patients; FT$_3$, free T$_3$; FT$_4$, free T$_4$; TRAb, anti-thyrotropin receptor antibody; TSAb, thyroid stimulating antibody; ns, not significant |
might be a potential candidate gene contributing to the
development of GD or influencing its clinical severity
and course. There have been four reports on the associ-
ation of the SNP of the CD40 gene with GD suscep-
tibility [15-18]. The first original study showed that
the C allele frequency was increased in GD in hetero-
genous populations of Caucasians (North America,
Italy, UK and Israel) [15]. Kim et al. [16] reported an
increased C allele frequency in GD in Korean popu-
lation. Two subsequent studies could not detect signif-
icant difference in allele or genotype frequency of the
CD40 SNP between GD and control subjects in UK
Caucasians, despite adequate power to detect an effect
[17, 18]. Heward et al. [18] conjectured on the reasons
behind the lack of replication. First, the positive result
could be due to a random chance event because of the
small sample size used in the original study. Second,
the original positive finding could be the result of the
“first time effect” phenomenon whereby association of
a gene is overestimated when first detected. Third, the
differences could be arising because of the different
ethnic, racial and geographical background of the pop-
ulations used in each study. They avoided the issues of
ethnic, racial and geographical diversity by using a
homogeneous population of UK Caucasians. However,
they could not find any association of the CD40 SNP
with GD susceptibility. There was no difference in the
genotype frequencies of the CD40 gene polymorphism
in control groups between the Korean and the Japanese,
suggesting that both have similar genetic backgrounds.
However, we could not confirm the significant increase
in C allele frequency in GD patients or patients with
ophthalmopathy. The number of samples was greater
in Japanese than that in Korean.

In the present study, however, we evaluated the dif-
ferences between the frequencies of CD40 gene alleles
in patients with GD onset before 40 years of age and
those with a later onset of GD. The associations be-
tween age at onset and different human leukocyte anti-
gen genotypes [28, 29] and ICAM-1 gene alleles [30]
have been reported. The criteria we used to stratify the
patients into those with early and later ages of onset of
disease were based on the epidemiological observa-
tions of the highest risk of the onset of GD [2]. Indeed,
we showed that there were two peaks in the distribution
of age at onset of GD. This suggests that at least two
factors may influence the GD susceptibility. There-
fore, we stress that age at onset of GD should be con-
sidered in the analysis. Although the number of
patients with TT genotype was small in the present
study, the TT genotype frequency was significantly
smaller in patients who developed GD after 40 years
old than those before 40 years old and control subjects.
The significance was also observed when female pa-
tients were analyzed. These findings raise the question
as to how this genetic polymorphism contributes to the
pathogenesis of GD in later onset GD. First, the possi-
bility that the SNP induces higher transcriptional ac-
tivity is considered. The second possibility is that
undefined genetic polymorphisms in linkage disequi-
librium with the CD40 SNP exist in other regions of the
CD40 gene or near loci of the gene. Third, unknown
factors associated with aging may influence the func-
tions of the CD40/CD40L interactions and result in
thyroid autoimmunity.

In conclusion, we found the association of CD40
SNP with GD susceptibility in Japanese GD patients
who developed GD after 40 years old. These results
suggest that CD40 SNP could influence the later onset
of GD in Japanese. Further studies in adequately sized
datasets in other populations and functional studies are
indicated.

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