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ORIGINAL ARTICLE

CD40 C/T_{-1} and CTLA-4 A/G_{49} SNPs are associated with autoimmune thyroid diseases in the Chinese population

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Abstract This study was to investigate whether the common polymorphisms of CD40 and CTLA4 genes confer susceptibility to AITD in the Chinese population. A set of unrelated subjects including 303 GD patients, 208 HT patients, and 215 matched healthy controls were recruited. SNPs were genotyped by the method of PCR-RFLP. (1) As for CD40 C/T_{-1} SNP, only a significant difference was found in allele frequencies between GD and control groups ($P = 0.033$). (2) On the part of CTLA-4 A/G_{49} SNP, significant differences were found in genotype and allele frequencies between GD and control groups ($P = 7.0 \times 10^{-5}$ and $P = 0.002$, respectively), and similar results were found between HT and control groups ($P = 0.015$ and $P = 0.003$, respectively). (3) The logistic regression analysis showed there was no interaction between CD40 and CTLA4 genotypes ($P = 0.262$). These results indicate that both CTLA-4 A/G_{49} and CD40 C/T_{-1} SNPs are associated with genetic susceptibility of GD, and CTLA-4 A/G_{49} is also associated with HT.

Keywords Graves' disease · Hashimoto's thyroiditis · CD40 · CTLA-4 · Single nucleotide polymorphisms (SNPs)

Jing Yang and Qiu Qin contributed equally in this study.

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Introduction

Autoimmune thyroid diseases (AITDs) are a group of autoimmune disorders that mainly include Graves' disease (GD) and Hashimoto's thyroiditis (HT). AITDs arise due to an interplay between environmental and genetic factors. Multiple genes and environmental factors have been identified and characterized for the susceptibility to AITD. These are immunoregulatory genes like the HLA Class II gene, the cytotoxic T lymphocyte-associated factor 4 (CTLA-4) gene, the CD40 gene and the protein tyrosine phosphatase-22 (PTPN22) gene, and others thyroid-specific genes for the TSH receptor and thyroglobulin. During antigen presentation, the co-stimulatory signals (e.g., CD-40, B7-1, and B7-2) are provided by antigen presenting cell (APCs), and interact with receptors (e.g., CD-40L, CD28, and CTLA-4) on the surface of CD4 + T cell. The process is important for the activation of T cells. It has been thought that the C allele of CD40 could increase humoral autoimmunity [1]. Case–control studies in different ethnic backgrounds showed an association between the CC genotype of CD40 Kozak single nucleotide polymorphism (SNP) and GD [2, 3]. However, this CD40 gene polymorphism did not detect any association with GD in Taiwanese [4]. On the other hand, researchers have shown an association between the A allele of CTLA-4 A/G_{49} SNP and increased CTLA-4 function and expression [1], while, reduced function and expression of CTLA-4 might augment autoimmunity. In association with AITD, the G allele of the A/G_{49} SNP has been reported to have different susceptibility variant in different ethnic groups [5–7]. A study in Hong Kong confirmed that the GG genotype has been associated with GD in Chinese children [8].

And a recent research with a large sample in the combined Chinese Han showed that CTLA-4 A/G_{49} is a susceptibility variant for GD [9]. To our knowledge, there has been no such study conducted suggesting that CD40 gene polymorphism may be associated with GD and HT in the Mainland China, and yet no any related study has been conducted about association between CTLA-4 A/G_{49} SNP and HT in the Mainland China, too. So in our study we screened for the polymorphisms of CD40 and CTLA-4, and detected the relationship of these genes with the susceptibility to AITD in the Chinese population.

Materials and methods

Experimental subjects

We investigated 511 non-related Chinese Han AITD patients, consisting of 303 GD patients (78 males and 225 females; aged 4–72 years, 34.14 ± 12.23 years) and 208 HT patients (12 males and 196 females; aged 6–64 years, 33.11 ± 12.06 years). All of them were treated in the outpatient department of Endocrinology Department in the First Affiliated Hospital of Xi'an Jiaotong University. The clinical data of the patients at the time of recruitment are summarized in Table 1. A total of 215 healthy controls (55 males and 160 females; aged 13–72 years, 34.76 ± 11.39 years) were selected from the Health Care Center from the same hospital. The diagnostic criteria for GD and HT were referred to our previously published article [10]. In brief, GD was diagnosed by clinical and biochemical assessment of hyperthyroidism, and the presence of diffuse goiter, the positive TPOAb or TgAb. Although TSH receptor antibody (TRAb) is positive in the majority of active GD, it is not a requisite marker for the diagnosis. HT was defined on the basis of enlarged thyroid, and the high level of either TPOAb or TgAb, with or without clinical and biochemical hypothyroidism. For the Suspicious cases of HT, diagnoses were confirmed by fine needle aspiration biopsies (FNAC). The serum levels of thyroid hormones and TPOAb and TgAb were determined by radioimmunoassay (Tianjin CO. LTD, Medical and Pharmaceutical Union Technology, Tianjin, China).

As it has been indicated that the genetic background of underlying thyroid associated ophthalmopathy (TAO) may be different from that of pure AITD, patients with overt ophthalmopathy were excluded in our patient groups [11]. DNA was obtained from the 511 patients and the 215 controls. The control group had no any, personal or a family history of thyroid disease or other autoimmune diseases. All the subjects gave the informed consent, and the study was approved by the local ethics committee.

SNP genotyping

Genomic DNA was extracted from 1 ml whole blood from each patient and each control by standard procedure using a DNA extraction kit (TianGen Biotech CO. LTD, Beijing, China). Genotyping of the CD40 and CTLA4 SNP was performed by PCR-RFLP method. Primers for CD40 were referred to the published article [12], and primers for CTLA-4 were designed by PRIMER 5.0 software (Microsoft Corp, PREMIER Biosoft International). All the primers were synthesized by Beijing Genomics Institute (Beijing, China). These primers are as follows:

CD40

Forward-CCTCTTCCCCGAAGTCTTCC
Reverse-GAAACTCCTGCGCGGTGAAT

Table 1 The clinical data of patients when samples were collected

Groups	Thyroid function	*n* (%)	TPOAb positive, *n* (%)	TgAb positive, *n* (%)	Methimazole, mg/d	L-Thyroxine, μg/d
GD	Hyperthyroidism	122 (40.26)	94 (31.02)	45 (14.85)	10–30	–
	Subclinical hyperthyroidism	96 (31.68)	67 (22.11)	34 (11.22)	5–15	–
	Normal thyroid function	64 (21.12)	42 (13.86)	31 (10.23)	2.5–10	–
	Subclinical hypothyroidism	15 (4.95)	11 (3.63)	6 (1.98)	2.5	–
	Hypothyroidism	6 (1.98)	5 (1.65)	3 (0.99)	–	–
	In total	303 (100.00)	219 (72.28)	119 (39.27)	–	–
HT	Hypothyroidism	39 (18.75)	39 (18.75)	37 (17.79)	–	25–150
	Subclinical hypothyroidism	88 (42.31)	85 (40.87)	82 (39.42)	–	12.5–25
	Normal thyroid function	81 (38.94)	77 (37.02)	71 (34.13)	–	12.5–25
	In total	208 (100.00)	201 (96.63)	190 (91.35)	–	–

n number; *d* day; *GD* Graves' disease; *HT* Hashimoto's thyroiditis; *TPOAb* anti-thyroid peroxidase antibody; *TgAb* thyroglobulin antibody

CTLA4

Forward-GAAGGATGGTGCTTCACAGAT
Reverse-CTTTGCAGA AGACAGGGATGA

PCRs were carried out in a total volume of 20 μl reaction mixture, containing 0.2 μg genomic DNA, 10 μl 2× Taq PCR Masterix (TianGen Biotech CO. LTD, Beijing, China), and 1 μl primer (10 μmol/l). PCR process included initial denaturation at 95°C for 5 min followed by 35 cycles including denaturation at 95°C for 30 s, annealing at 67°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min for the samples for CD40 SNP. And for the samples of CTLA4 SNP initial denaturation at 94°C for 3 min followed by 37 cycles including denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. After amplification, products of CD40 were digested with the restriction enzyme *Sty*I (Fermentas Life Sciences) at 37°C for 12 h, the size of the fragments was 74, 99, and 129 bp for CC genotype; 99 and 203 bp for TT genotype; 74, 99, 129, and 203 bp for CT genotype, respectively. While the amplified products of CTLA4 were digested with the restriction enzyme *Bbv*I (Fermentas Life Sciences) at 65°C for 4 h, the fragments were 96 and 556 bp for AA genotype; 76, 96, and 480 bp for GG genotype; 76, 96, 480, and 556 bp for AG genotype in size, respectively. Then the products were analyzed on 3% agarose gel.

Statistical analysis

Genotype frequencies for both SNPs were tested for Hardy–Weinberg equilibrium using SHEsis software platform. Case–control analysis was performed using chi-square test, and interaction was analyzed by logistic regression analysis using the SPSS 17.0. A P value of <0.05 was considered significant.

Results

The genotype frequencies for CTLA-4 A/G_{49} and CD40 C/T_{-1} SNPs were in Hardy–Weinberg equilibrium in control groups ($P = 0.098$ and $P = 0.753$). The distribution of genotypes and allele frequencies of CD40 C/T_{-1} in AITD and controls are presented in Table 2. There was significant difference in allele frequencies ($\chi^2 = 4.538$, $P = 0.033$), but not in genotype frequencies ($\chi^2 = 5.687$, $P = 0.058$) between GD and control groups. However, there was no any significant difference seen in genotype or allele frequencies between the HT and control groups ($\chi^2 = 1.824$, $P = 0.402$ and $\chi^2 = 0.742$, $P = 0.389$, respectively).

The distribution of genotypes and allele frequencies of CTLA-4 A/G_{49} in AITD and controls are presented in Table 3. Both genotype and allele frequencies were significantly different ($\chi^2 = 16.519$, $P = 7.0 \times 10^{-5}$ and $\chi^2 = 9.896$, $P = 0.002$, respectively) between GD and control groups. Similar results were found between HT and control groups ($\chi^2 = 8.395$, $P = 0.015$ and $\chi^2 = 8.601$, $P = 0.003$, respectively).

The effect of CD40 and CTLA-4 genotype on GD is presented in Table 4. Logistic regression analysis revealed no interaction between CD40 and CTLA-4 genotype on GD ($P = 0.262$).

Discussion

CD40 is expressed not only on classical APCs such as dendritic cells, but also on non-terminally differentiated B-cells as signal receptor in B-cell activation [13]. A B-cell expressing a higher level of surface CD40 may be expected to have a lower threshold for activation. Indeed, there are reports documenting that even a modest change in the expression levels of B-cell surface receptors can precipitate an autoimmune condition [1]. A study found that the C allele increased the expression of CD40 protein by enhancing the gene translation efficiency [14]. Actually, CD40 over-expression has been found on dendritic cells in GD [15]. An evidence for involvement of multiple cell types in autoimmune disease has shown that CD40 is

Table 2 Genotype distribution and allele frequencies of CD40 C/T_{-1} in AITD patients and controls

	GD ($n = 303$)	HT ($n = 208$)	Control ($n = 215$)	GD vs. control	HT vs. control
Genotype frequency					
CC	147 (48.5)	81 (38.9)	87 (40.5)	$\chi^2 = 5.687$	$\chi^2 = 1.824$
CT	141 (46.5)	99 (47.6)	108 (50.2)	$P = 0.058$	$P = 0.402$
TT	15 (5.0)	28 (13.5)	20 (9.3)		
Allele frequency					
C	435 (71.8)	261 (65.3)	282 (65.6)	$\chi^2 = 4.538$	$\chi^2 = 0.742$
T	171 (28.2)	155 (34.7)	148 (34.4)	$P = 0.033$	$P = 0.389$
				OR = 1.335	
				(1.023, 1.742)	

Table 3 Genotype distribution and allele frequencies of CTLA-4 A/G_{49} in AITD patients and controls

	GD ($n = 303$)	HT ($n = 208$)	Control ($n = 215$)	GD vs. control	HT vs. control
Genotype frequency					
AA	12 (4.0)	17 (8.2)	29 (13.5)	$\chi^2 = 16.519$	$\chi^2 = 8.395$
AG	139 (45.9)	77 (37.0)	97 (45.1)	$P = 7.0 \times 10^{-5}$	$P = 0.015$
GG	152 (50.1)	114 (54.8)	89 (41.4)		
Allele frequency					
A	163 (27.4)	111 (26.8)	155 (38.0)	$\chi^2 = 9.896$	$\chi^2 = 8.601$
G	443 (72.6)	305 (73.2)	275 (62.0)	$P = 0.002$	$P = 0.003$
				OR = 1.532	OR = 1.549
				(1.174, 2.000)	(1.155, 2.076)

Table 4 Effect of CD40 and CTLA -4 genotype on GD

Genotype		GD ($n = 303$)	Control ($n = 215$)	CD40 by CTLA4 $P = 0.262$
CD40	CTLA-4			
TT + CT	AA + AG	67	73	
TT + CT	GG	89	59	
CC	AA + AG	84	53	
CC	GG	63	30	

upregulated on human thyroid follicular cells in GD [16]. Different ethnic backgrounds studies, for example, Caucasian GD patients in 2002 [11] and 2005 [2], Korean GD patients in 2003 [17], Japanese GD patients in 2005 [3] and 2006 [18], and a meta-analysis in 2005 showed a significant association between the CC genotype and GD [2]. While, in a different aspect, the study on the Chinese population did not find these association in Taiwanese in 2008 [4]. Moreover, CD40 C/T_{-1} was not found to be associated with HT in Koreans [15] or in Japanese [16]. Our case–control study has shown an association of CD40 C/T_{-1} SNPs with AITD in the Chinese Han population, that C allele raises the risk of GD, but not HT.

CTLA-4, a 188 amino acid glycoprotein, is expressed on activated T cells [19]. Its ligation with either the B7-1 or B7-2 can initiate its signal, thus playing a role as a negative regulator in T-cell-mediated immune response. In addition, CTLA-4 knockout mice have suggested that CTLA-4 suppresses and terminates response of T cell and is crucial for control of lymphocyte homeostasis [20]. The A/G_{49} SNP results in Thr > Ala substitution, and researchers found that the polymorphism reduced the inhibitory function of CTLA-4, and the G allele reduced the ability to control T-cell proliferation [21]. The studies in Caucasian GD patients in 1995 [6], Japanese GD patients in 1997 [5] and 2002 [22], Korean GD patients in 2000 [7], and the combined Chinese Han in 2010 [9] showed it is a susceptibility variant for GD. On the part of HT, studies in Korean HT patients in 2000 [7] and Caucasian HT patients in 2002 [23] showed that the gene is not only specific to GD but also confers susceptibility to HT. Furthermore, studies have suggested that CTLA-4 A/G_{49} may play a role in the severity of the AITD phenotype like patients with more severe thyrotoxicosis at diagnosis [24], or who could not achieve remission over 5 years after anti-thyroid medications [25]. A meta-analysis suggested significant associations with GD and HT for CTLA-4 A/G49 SNP in both Asian and Caucasian descent subjects [26]. However, studies in Turkish population showed this polymorphism was just associated with GD [27], but not HT [28]. Our study has shown that the CTLA-4 A/G_{49} SNP is predisposed to the development of AITD in the Chinese Han population, and G allele raised the risk of GD and HT.

To sum up, we have confirmed that the C allele of CD40 C/T_{-1} SNP plays a role in the pathogenesis of GD, and that the G allele of CTLA-4 A/G_{49} SNP plays a role in AITD. However, we failed to find an overlapping or synergic effect between the two SNPs. The complicated relationship between them remains to be further elucidated.

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