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ORIGINAL PAPER

Association of the thyroglobulin gene polymorphism with autoimmune thyroid disease in Chinese population

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Abstract *Objective* This study was performed to identify the presence of previously reported thyroglobulin (Tg) gene single nucleotide polymorphisms (SNPs) in Han Chinese Asians, and to investigate their potential relation to autoimmune thyroid disease (AITD). *Methods* Polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) in 228 Chinese patients with AITD (146 with Graves' disease and 82 with Hashimoto's thyroiditis) and 131 healthy Chinese controls. *Results* (1) The occurrence of four common Tg gene SNPs (E10SNP24 T/G and E10SNP158 T/C in exon 10, E12SNP A/G in exon 12, and E33SNP C/T in exon 33) was confirmed in this Chinese population. No differences in allele and genotype frequencies were found between AITD patients and control subjects, or between male and female individuals in any group. Neither were differences in allele frequencies observed when Graves' disease (GD) or Hashimoto's thyroiditis (HT) patients were analyzed separately. (2) Haplotype analysis of these four SNPs revealed that the G-C-A-C haplotype was significantly associated with HT ($P < 0.01$, OR = 3.06, OR 95% CI [1.326–7.089]) and with serum anti-Tg antibody (Tg-Ab) positive AITD patients ($P = 0.028$, OR = 3.34). *Conclusion* Our study confirms the existence of four SNPs among Han Chinese. In addition, the association of one SNP haplotype with HT suggests that Tg may be an AITD susceptibility gene.

Keywords Graves' disease · Hashimoto's thyroiditis · Thyroglobulin · Single nucleotide polymorphism (SNP)

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Introduction

Autoimmune thyroid diseases (AITDs), including Graves' disease (GD) and Hashimoto's thyroiditis (HT), are among the most common autoimmune diseases worldwide. The incidence of AITD over the past decades has increased dramatically in China, mirroring observations in the western countries, where irregular and aggressive iodine supplementation has been postulated to play an etiologic role [1–3]. Epidemiologic evidence, including strong familial aggregation, supports a genetic contribution to the development of AITD. Relative to the background population, siblings of AITD patients are estimated to exhibit a 10–15-fold greater risk of developing clinical disease [4], and monozygotic twins exhibit a higher rate of concordance for overt and subclinical disease (positive for antithyroid antibodies) compared to dizygotic twins [5, 6].

However, although many genes have been investigated, only two genes, HLA-DR3 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), have been consistently linked to AITD across populations [7–9]. It is unlikely that these two immune regulatory genes fully account for disease development. Thyroid stimulating hormone receptor (TSHR), thyroid peroxidase (TPO), and Tg are the main autoantigens of AITD. Thus far, international and Chinese investigators have failed to demonstrate an association between TSHR or TPO and AITD. However, data concerning the Tg gene obtained from various populations has provided more controversial results [10–12]. Because the ethnic heterogeneity may influence the association of AITD with a specific gene, we performed a study of the four Tg gene SNPs in Chinese population. Our data indicate that one of the SNPs is associated with AITD.

Results

Detection of SNPs

PCR amplification of exon 33 produced a replicon of 411 bp. Digestion of the homozygous TT genotype of E33SNP with *HpyCH4 III* produced two fragments of 275 bp and 136 bp. While digestion of the heterozygous TC genotype with the same enzyme produced four fragments of 275 bp, 180 bp, 136 bp, and 95 bp, the digestion of the homozygous CC genotype produced three fragments of 180 bp, 136 bp, and 95 bp (Fig. 1). PCR amplification of exon 12 produced a replicon of 494 bp. Digestion of the homozygous AA genotype of E12SNP with *BsaA I* produced two fragments of 401 bp and 93 bp. Digestion of the homozygous GG genotype with the same enzyme produced three fragments of 248 bp, 153 bp, and 93 bp, while digestion of the heterozygous TC genotype produced four fragments (401 bp, 248 bp, 153 bp, and 93 bp) (Fig. 2). PCR amplification of exon 10 produced a replicon of

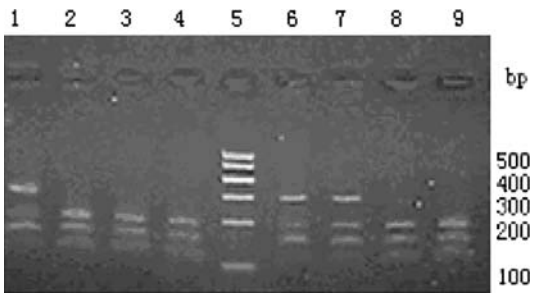

Fig. 1 Restriction digests of E33SNP PCR products. Following PCR amplification of Tg exon 33 products were digested with *HpyCH4 III*. Digests were electrophoresed on a 3% agarose gel and exposed with ETBR and UV light. Lanes 1, 6 and 7 reveal the TC genotype, lanes 2, 3, 4, 8, and 9 the CC genotype, and lane 5 the 100 bp DNA marker

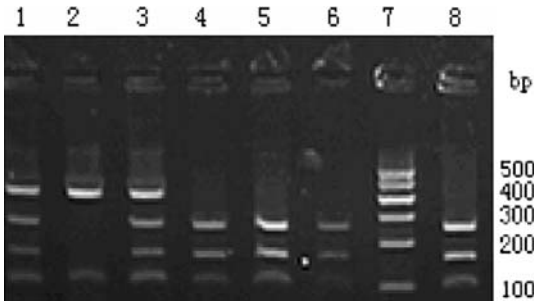

Fig. 2 Restriction digests of E12SNP PCR products. Following PCR amplification of Tg exon 12 products were digested with *BsaA I*. Digests were electrophoresed on a 3% agarose gel and exposed with ETBR and UV light. Lanes 1 and 3 reveal the AG genotype, lane 2 the AA genotype, lanes 4, 5, 6, and, 8 the GG genotype and lane 7 the 100 bp DNA marker

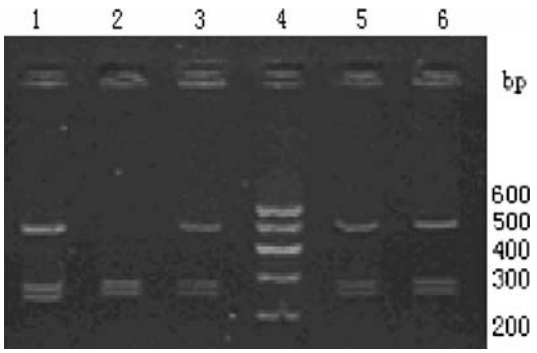

Fig. 3 Restriction digests of E10SNP PCR products with *Blp I*. Digests were electrophoresed on a 3% agarose gel and exposed with ETBR and UV light. Lanes 1, 3, 5, and 6 reveal the TC genotype, lane 2 the GG genotype, and lane 4 the 100 bp DNA marker

452 bp. Digestion of the homozygous GG genotype of E10SNP *Blp I* produced two fragments of 237 bp and 215 bp, while the digestion of the heterozygous TG genotype produced three fragments of 452 bp, 237 bp, and 215 bp (Fig. 3). Digestion of the homozygous CC genotype of E10SNP with *Ava I* produced two fragments of 368 bp and 84 bp. Digestion of the heterozygous TC genotype, also with *Ava I,* produced three fragments of 452 bp, 368 bp, and 84 bp (Fig. 4).

Case–control study

All genotypes were in Hardy–Weinberg equilibrium. Table 1 shows the frequencies of these alleles in AITD patients and controls. The frequencies of all alleles and genotypes were not significantly different between AITD patients and controls. When all groups were stratified according to sex, the frequency of these SNPs was not significantly different between males and females in any group (data not shown). The result of the case–control association analysis is shown in Table 2. There was no

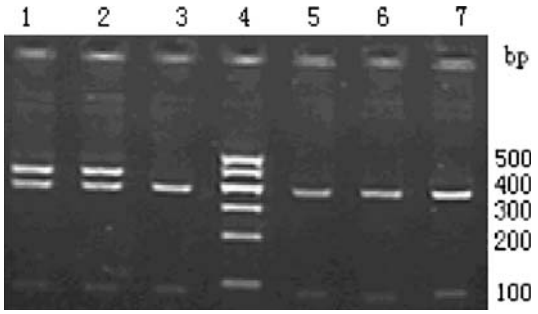

Fig. 4 Restrction digests of E10SNP PCR products with *Ava I*. Digests were electrophoresed on a 3% agarose gel and exposed with ETBR and UV light. Lanes 1 and 2 reveal the TC genotype, lanes 3, 5, 6, and 7 the GG genotype, and lane 4 the 100 bp DNA marker

evidence of an association between any of the SNPs and AITD. Subset analysis demonstrated that these four SNPs were not associated with either GD or HT.

Linkage disequilibrium among the SNPs

Table 3 presents the pair-wise linkage disequilibrium (LD) values calculated for SNPs. LD for each SNP in controls was expressed as D′. A strong LD was detected between E10SNP24 and E10SNP158 ($D' = 0.96$, $r^2 = 0.906$). A weak LD was also detected between E12SNP and E10SNP24, E12SNP and E10SNP158, $D' = 0.732$ and 0.739, respectively, and $r^2 = 0.458$ and 0.462, respectively.

Table 2 Case–control analysis of Tg SNPs in GD and HT patients

SNP	Alleles χ^2	*P* value	Genotypes χ^2	*P* value
GD				
E33SNP	0.0976	0.7546	0.2113	0.899
E12SNP	0.0067	0.9349	1.4244	0.4906
E10SNP24	2.9532	0.0858	3.8470	0.1462
E10SNP158	1.3607	0.2436	2.1016	0.3497
HT				
E33SNP	0.5758	0.4479	0.5141	0.77335
E12SNP	0.6407	0.4773	1.4794	0.4235
E10SNP24	2.1211	0.1454	2.9234	0.2319
E10SNP158	1.4198	0.2335	2.6313	0.2683

Table 3 Linkage disequilibrium test between Tg SNPs in control subjects

Tg SNPs	E12SNP	E10SNP24	E10SNP158
E33SNP	0.106	0.204	0.109
	E12SNP	0.732	0.739
		E10SNP24	0.964

Haplotype analysis of the SNPs

The haplotype distribution was calculated for each of the four SNPs, and case–control haplotype association analysis was performed in patients and controls as shown in

Table 1 Allele and genotype frequencies of the Tg SNPs in AITD patients and control subjects

SNP	Alleles/genotypes	AITD [number(%)]	Control [number(%)]	χ^2	*P*	OR	OR 95% CI
E10SNP24	T	102 (22.4)	53 (28.6)	3.508	0.072	0.70	0.49–1.01
	G	354 (77.6)	187 (71.4)	4.162	0.125	0.79	0.26–2.41
	TT	7 (3.1)	6 (4.6)				
	TG	88 (38.6)	63 (48.1)				
	GG	133 (58.3.)	62 (47.3)				
E10SNP158	T	102 (22.4)	72 (27.4)	2.369	0.125	0.77	0.54–1.11
	C	354 (77.6)	190 (72.6)	4.080	0.130	1.02	0.35–2.95
	TT	11 (4.9)	6 (4.8)				
	TC	85 (35.1)	60 (45.2)				
	CC	137 (60.0)	65 (50.0)				
E12SNP	A	125 (27.5)	143 (28.7)	0.164	0.686	1.06	0.74–1.50
	G	329 (72.5)	255 (71.3)	1.326	0.515	0.69	0.27–1.75
	AA	11 (4.8)	9 (3.6)				
	AG	103 (45.4)	125 (50.2)				
	GG	113 (47.8)	115 (46.2)				
E33SNP	C	351 (77.0)	202 (77.1)	0.018	1.00	1.02	0.69–1.50
	T	105 (23.0)	60 (22.9)	0.444	0.801	1.06	0.65–1.73
	CC	142 (62.3)	80 (61.1)				
	CT	67 (29.4)	42 (32.1)				
	TT	19 (8.3)	9 (6.9)				

Table 4 Association of genotype combination in the Tg gene with AITD

Haplotype	AITD [number(%)]	Control [number(%)]	χ^2	*P*	OR	OR 95% CI
T-T-A-C	46 (14.4)	32 (15.7)	0.162	0.688	0.91	0.56–1.47
G-C-A-C	23 (7.2)	9 (4.4)	1.650	0.199	1.66	0.76–3.65
T-T-G-C	7 (2.5)	10 (5.2)	2.739	0.098	0.46	0.18–1.76
G-C-G-C	171 (53.4)	104 (50.1)	0.526	0.468	1.14	0.80–1.61
G-C-G-T	49 (15.3)	34 (16.7)	0.662	0.660	0.90	0.56–1.44

Table 5 Association of genotype combination in the Tg gene with HT

Haplotype	HT [number(%)]	Control [number(%)]	χ^2	*P*	OR	95% CI
T-T-A-C	20 (15.7)	32 (15.7)	0.000	0.997	0.67	0.55–1.82
G-C-A-C	16 (12.5)	9 (4.4)	7.444	0.006	3.07	1.33–7.09
T-T-G-C	3 (2.6)	10 (5.2)	1.296	0.255	0.50	0.95–1.69
G-C-G-C	64 (48.7)	104 (50.1)	0.064	0.801	0.95	0.61–1.46
G-C-G-T	21 (16.0)	34 (16.7)	0.029	0.8657	0.95	0.53–1.72

Table 6 Association of genotype combination of SNPs of Tg gene with Tg-Ab in AITD

Haplotype	Tg-Ab positive [number(%)]	Tg-Ab negative [number(%)]	χ^2	*P*	OR	OR 95% CI
T-T-A-C	28 (15.6)	14 (13.0)	0.392	0.531	1.24	0.63–2.45
G-C-A-C	19 (10.4)	3 (3.4)	1.84	0.028	3.34	1.08–10.32
G-C-G-C	89 (48.4)	67 (60.3)	3.904	0.053	0.62	0.39–0.99
G-C-G-T	33 (18.1)	12 (11)	2.662	0.103	1.782	0.88–3.59

Table 4. In order to investigate whether particular combinations confer a strong susceptibility to AITD, interactions between these SNPs were also analyzed. Five common haplotypes, T-T-A-C, G-C-A-C, T-T-G-C, G-C-G-C, and G-C-G-T, were found, but an association with AITD as a group was demonstrated for none of them. However, when GD and HT subjects were analyzed separately, we found that the G-C-A-C haplotype was significantly more prevalent in the HT group ($P < 0.01$, OR = 3.066, 95% CI [1.326–7.089]) (Table 5).

Association of Tg-Ab with SNPs

Tg-Ab was routinely measured in all AITD patients, and 142(62%) of them were positive. No difference in the frequencies of the four SNPs was found between Tg-Ab positive and negative AITD patients (data not shown). However, when case–control haplotype association analysis was performed in these two subgroups, we found that the G-C-A-C haplotype was significantly more common among Tg-Ab positive subjects ($P = 0.028$, OR = 3.345, OR 95% CI [1.08–10.32], and the G-C-G-C haplotype was significantly less common in this group ($P = 0.048$, OR = 0.6, OR 95% CI [0.39–0.99]) (Table 6).

Discussion

Tg is a major thyroid-specific autoantigen. AITDs are characterized by cellular and humoral immune responses to Tg. Indirect evidence supports contentions that Tg plays an important role in the etiology of AITD. High serum titers of Tg-Ab are common in patients with AITD and in animal models of spontaneous autoimmune thyroiditis [13]. Tg immunization induces experimental autoimmune thyroiditis (EAT) in mice in an MHC dependent manner, implying an interaction between the Tg and the MHC molecules in the induction of thyroiditis [14]. Tg specific auto-reactive T lymphocytes are capable of transferring EAT passively from one mouse to another [15]. Tg-Ab from AITD patients is characterized by restricted epitope specificity while Tg-Ab from healthy subjects are polyclonal, and recognize Tg antigenic domains distinct from those recognized by Tg-Ab from AITD patients [16] .

Recent evidence indicates a direct contribution of the Tg gene toward an AITD predisposition. A genome-wide scan performed in 123 Japanese sibling pairs revealed linkage of a marker (D8S272) in the Tg region on chromosome 8q24 with AITD [17]. This linkage relationship was replicated in 102 multigenerational Caucasian pedigrees [18]. Further,

the Tg gene region was fine mapped to the Tg gene itself [19]. Most recently, all 48 exons of the Tg gene have been resequenced, and novel single nucleotide polymorphisms (SNPs) have been identified. Case–control association studies for 14 Tg SNPs indicated that one SNP cluster (the exons 10–12 cluster) and an exon 33 SNP were significantly associated with AITD in an American Caucasian population. Moreover, about half of 19 EAT-susceptible mice possessed haplotypes involving exon 10 and 12 SNPs, which were not present in any of the mouse strains that were resistant to thyroiditis [12]. Other work has refuted these findings. These four SNPs were found to have no association with AITD in a U.K. Caucasian population [10]. Other investigation showed that exon 33 SNP was not associated with HT in a Japanese population [11] .

This is the first analysis of the relationship between the Tg gene and AITD among Han Chinese. We have confirmed the presence of SNPs previously reported in Chinese (E10SNP24T/G, E10SNP158T/C, E12SNPA/G, E33SNPC/T). When a case–control study was performed using these 4 SNPs, we found no significant association between individual SNP and AITD, GD, or HT. However, when we carried out an analysis for SNP combinations, we found that one of the five common haplotypes was associated with HT susceptibility. This finding supports the previous reports in American Caucasian subjects that the Tg gene is a susceptibility gene for HT.

The mechanism by which the Tg gene may predispose to AITD remains unknown. The additional finding in this study that Tg seems to be involved in the production of Tg autoantibody in AITD patients suggests that missense SNPs in the Tg gene may enhance the antigenicity of the Tg protein [20]. However, while thyroglobulin is clearly a major thyroid-specific antigen, there is no evidence that the corresponding antibodies cause tissue or cell damage in AITD [21]. On the other hand, several groups have shown that TPO antibodies are cytotoxic to thyroid cells and are more likely to play a pathogenic role in patients with HT [22, 23]. Thus, the Tg gene may be a marker for autoimmunity in humans, but not directly linked to its cause.

In conclusion, the current study supports an association between haplotypes of the SNPs at exon 10, 12 and 33 and susceptibility to HT. These SNPs may be involved in enhancing immunogenicity of Tg and the production of Tg autoantibody. Tg was confirmed as a susceptibility gene for HT in Han Chinese population.

Material and methods

Subjects

A total of 228 unrelated Chinese patients with AITD (mean age 36.66 ± 14.15 years) were consecutively recruited from the endocrinology clinics of the First Affiliated Hospital of the Medical School, Xi'an Jiaotong University and 131 healthy Chinese (30 men and 101 women, mean age 34.6 ± 13.30 years) matched with patients for age, gender, and ethnicity served as controls. All the control subjects had neither a personal or a family history of any autoimmune disease nor thyroid goiter. Patients were classified according to clinical and biochemical assessment as having AITD, and were subclassified as having either GD ($n = 146$, 32 men and 114 women, mean age 37.33 ± 14.39 years) or HT ($n = 82$, 16 men and 66 women, mean age 35.51 ± 13.76 years). AITDs were diagnosed based on previously published clinical and laboratory findings [20]. Briefly, GD was defined by biochemical confirmation of hyperthyroidism, and two of the following criteria: diffuse goiter, thyroid-related eye disease, or a combination of positivity for anti-thyroid stimulating hormone receptor antibody (TRAb), anti-thyroid peroxidase antibody (TPO-Ab), and anti-thyroglobulin antibody (Tg-Ab). HT was diagnosed based on the presence of an enlarged thyroid and the presence of either TPO-Ab or Tg-Ab, with or without documented clinical and biochemical hypothyroidism. Most diagnoses of HT were confirmed by fine needle aspiration biopsies. Based on serum Tg-Ab detection, all the AITD patients were subdivided into a Tg-Ab positive group ($n = 142$) and a Tg-Ab negative group ($n = 86$) and evaluated in case–control analysis. All antibodies were measured by radioimmunoassay, and kits were obtained commercially from Tianjin CO. LTD, Medical and Pharmaceutical Union Technology (Tianjin, China). A level of higher than 30 ng/ml was considered positive for Tg-Ab assay.

Informed consent was obtained from all subjects, and the study was approved by the local ethics committee.

PCR and restriction digestion

The sequences of Tg exons 10, 12, and 33 were obtained from GenBank [exon 10 accession nos. X06069, exon 12 accession nos. AF170486, and exon 33 accession nos. AF169662] accessed through www.ncbi.nlm.nih.gov, and aligned to Homo sapiens chromosome 8q24.3 clone [accession nos. AF230667 (exon 10 and exon 12) and AF305872 (exon 33)] using a basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/). We designed primers using chromosome 8q24.3 sequences in PRIMER 5.0 software (1990–2000 Microsoft Corp, PREMIER Biosoft International). Primers were synthesized by the AuGCT Biotechnology CO. LTD. (Beijing, China). Primers sequences are shown in Table 7.

DNA was extracted from the whole blood of subjects using the Nucleon Bacc kit from TianGen Biotech CO. LTD. (Beijing, China). Target DNA fragments were

Table 7 Primers for the exons

SNP	Primer
E33SNP	F 5′ ATC CAC TCA TGC ATA TTG ACC A 3′
	R 5′ AGC CAT GTC TCA GCT ACC ACA 3′
E12SNP	F 5′ TAC TGT GCA AGG AGC CTT TGT 3′
	R 5′ GGC TGG TAG AAG TTT CCT GCT 3′
E10SNP24	F 5′ CTG CAG TGC TGA TCA CCA ACT 3′
	R 5′ ATC TTC ACT AGC AGC TTG GCA 3′
E10SNP158	F 5′ CTG CAG TGC TGA TCA CCA ACT 3′
	R 5′ ATC TTC ACT AGC AGC TTG GCA 3′

amplified by PCR using appropriate primers and under optimal conditions as stated below.

For exon 10, PCR reactions were performed in a final volume of 25 μl, containing 1.5 μl genomic DNA and 1 μl (10 μM) of each primer, 12.5 μl 2 × Taq PCR MasterMix (TianGen Biotech CO. LTD, Beijing, China). The mixture contained 0.1U/μl Taq Polymerase, 500 μM of each dNTP, 20 mM Tris-HCl (PH8.3), 100 mM KCl, 3 mM $MgCl_2$, and other intensifiers. Reaction mixtures were denatured at 94°C for 3 min and then cycled 30 times as follows: 30 s at 94°C for denaturing, 30 s at 56°C for annealing, and 30 s at 72°C for extension. A final extension step at 72°C for 5 min was then performed. For exon 12, PCR conditions and cycling parameters were the same as those for exon 10, except that an annealing step of 57°C was used. For exon 33, PCR condition and cycling parameters were the same as for exon 10, except that an annealing step of 55°C was used.

All PCR products were digested using previously described restriction enzymes [10]. The enzymes *HpyCH4 III, BsaA I*, and *Blp I* were purchased from New England BioLabs CO. LTD (Beijing, China); *Ava I* was from Takara Biotechnology CO. LTD (Dalian, China). All digested PCR products were separated by electrophoresis on 3% agarose gels and visualized by ultraviolet light exposure following staining with ethidium bromide.

Statistical analysis

Case–control analysis of the data was performed using the χ^2 test (SPSS version 11.0) and Fisher's exact test with Yates correction. A P value of <0.05 was considered significant. Linkage disequilibrium between SNPs was evaluated by Lewontin's D′ (|D′|) [24] and r^2 using SHESIS software platform [25] on NCBI (http://www.nhgg.org/analysis/). A Full-Precise-Iteration (FPI) algorithm was used in haplotype reconstruction and frequency estimation using SHESIS software platform.

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