ORIGINAL

Association between thyroid stimulating hormone receptor gene intron polymorphisms and autoimmune thyroid disease in a Chinese Han population

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Abstract. Autoimmune thyroid disease (AITD) is a multifactorial disease with a genetic susceptibility and environmental factors. The thyroid stimulating hormone receptor gene (TSHR) which is expressed on the surface of the thyroid epithelial cell is thought to be the main auto-antigen and a significant candidate for genetic susceptibility to AITD. This case-control study aimed at evaluating the association between single nucleotide polymorphisms (SNP) of TSHR and AITD in a Chinese Han population. We recruited 404 patients with Graves' disease (GD), 230 patients with Hashimoto's thyroiditis (HT) and 242 healthy controls. The Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometer (MALDI-TOF-MS) Platform was used to detect five SNPs (rs179247, rs12101255, rs2268475, rs1990595, and rs3783938) in TSHR gene. The frequencies of allele T and TT genotype of rs12101255 in GD patients were significantly increased compared with those of the controls ($P=0.004/0.015$, $OR=1.408/1.446$). The allele A frequency of rs3783938 was greater in HT patients than in the controls ($P=0.025$, $OR=1.427$). The AT haplotype (rs179247-rs12101255) was associated with an increased risk of GD ($P=0.010$, $OR=1.368$). The allele A of rs179247 was associated with ophthalmopathy in GD patients. These data suggest that the polymorphisms of rs12101255 and rs3783938 are associated with GD and HT, respectively.

Key words: Thyroid stimulating hormone receptor (TSHR), Single nucleotide polymorphism (SNP), Autoimmune thyroid disease (AITD), Graves' disease (GD), Hashimoto's thyroiditis (HT)

AUTOIMMUNE THYROID DISEASE (AITD), a group of the most common organ-specific endocrine disease which could be typically represented by Graves' disease (GD) and Hashimoto's thyroiditis (HT), affects up to approximately 1% of the general population [1]. Although the fundamental pathogenesis of AITD remains unknown, it is generally acknowledged that genetic predispositions and environmental factors including exposure to cigarette smoke, high dietary iodine intake and stressful life events are implicated [2]. Concordance studies in twins suggest that genetic factors confer 80% contribution to the etiology of AITD [3]. In recent years, the genome-scanning and single nucleotide polymorphisms (SNP) studies

have made great progress in identification of susceptibility genes. Currently, several candidate genes have been reported, which include human leukocyte antigen (HLA) [4-6], cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) [7-9], thyroid stimulating hormone receptor (TSHR) [10-14], thyroglobulin (TG) [15, 16], protein tyrosine phosphatase (PTPN) gene [17, 18], CD40 gene [19, 20] and FCRL3 gene [21]. Among these genes, TSHR is deemed to be an important auto-antigen for thyroid and definitely plays a significant role in the pathogenesis of AITD [22].

TSHR is expressed on the thyroid follicular cell surface membrane, regulates thyroid growth, hormone synthesis and secretion physiologically by binding to TSH. However, in AITD patients, the body produces auto-antibodies including TSAb (thyroid-stimulating antibody) and TSBAb (TSH-stimulation blocking antibody) against TSHR, affecting the thyroid cell growth and differentiation and ultimately leading to thyroid dysfunction [23].

Submitted Jan. 16, 2012; Accepted Apr. 26, 2012 as EJ12-0024
Released online in J-STAGE as advance publication May 17, 2012

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Original studies on TSHR gene single nucleotide polymorphisms mainly concentrated on three polymorphic sites: two in exon1 (D36H and P52T) [24-26] and one in exon10 (D727E) [27, 28]. However, most of them hardly confirmed the correlations. We previously used polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) in patients with GD and HT to detect mutation in TSHR exon1, none mutation was still observed [29]. Ho *et al.* were the first to find the association of an intron1 SNP and C/G+63IVS1 with GD in a cohort of Singapore patients of multi-ethnic origins. Since then researchers have shifted focus to intronic SNPs study [10]. A two-stage case-control association study was conducted in two independent Caucasian data sets, suggesting that TSHR was the first replicated GD-specific locus but not autoimmune hypothyroidism. Moreover, they discovered that rs2268458 in intron 1 was the most associated GD SNP ($P=2\times 10^{-6}$, $OR=1.3$) [11]. In recent years, the association studies on AITD and TSHR intronic SNPs in Caucasian and Japanese cohorts all showed strong relevance, which provided us forceful motivation to research the relationship between TSHR intronic polymorphism and AITD in our ethnic groups. The case-control study in Japanese cohorts found that several SNPs spaced 3-50kb apart spanning the TSHR gene in intron7 and 8 were associated with GD, especially rs2268475 ($P=0.0004$), rs1990595 ($P=0.0086$) and rs3783938 ($P=0.0099$) [12]. More recently, a screening for 98 SNPs spanning about 800kb of TSHR gene in UK European ancestry found that 28 SNPs were associated with GD, in which the most relevant SNPs were rs179247 ($P=8.9\times 10^{-8}$, $OR=1.53$) and rs12101255 ($P=1.95\times 10^{-7}$, $OR=1.55$) [13]. They also selected several SNPs from intron7, but no association was observed. In 2010, an investigation in three European Caucasian cohorts validated rs179247 and rs12101255 displaying powerful association with GD cases; moreover, its logistic regression results suggested that rs12101255 was the major susceptibility locus [14].

In this study, we attempted to find the association of rs179247, rs12101255, rs2268475, rs1990595 and rs3783938 at the TSHR gene locus with GD or HT in a Han Chinese population. Furthermore, we analyzed the association between genotypes and AITD clinical characteristics.

Subjects and Methods

Subjects

All AITD (GD and HT) patients in the present case-control study were recruited from the Department of Endocrinology, the First Affiliated Hospital of Xi'an Jiaotong University. As Table 1 shows, our study investigated 634 AITD patients (men 22.40% and women 77.60%), who comprised 404 GD (men 28.21% and women 71.79%) and 230 HT patients (men 12.17% and women 87.83%). In GD patients, the average age of onset was 31.93 ± 13.86 , 74 individuals had family history and 101 had ophthalmopathy. In HT patients, the average age of onset was 29.92 ± 12.85 , 47 individuals had family history and 8 had ophthalmopathy. The diagnostic criteria for GD were mainly determined by clinical manifestation and laboratory biochemical proof of hyperthyroidism, and the presence of diffuse goiter, the positive circulating TSH receptor antibody (TRAb) or antibody against thyroglobulin (TGAb) or antibody against thyroid peroxidase (TPOAb). 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). Another 242 controls were recruited from unrelated physical examination individuals in the Health Check-up Center of the

Table 1 Clinical data of AITD patients and controls

	GD	HT	control
N	404	230	242
Gender			
Female	290(71.79%)	202(87.83%)	175(72.31%)
male	114(28.21%)	28(12.17%)	67(27.69%)
Age	34.21 ± 13.78	31.53 ± 12.89	34.84 ± 12.81
Onset of age	31.93 ± 13.86	29.92 ± 12.85	
Thyroid size			
Normal size	23(5.69%)	12(5.22%)	
I degree	41(10.15%)	21(9.13%)	
II degree	265(65.59%)	174(75.65%)	
III degree	75(18.57%)	23(10.00%)	
Family history			
(+)	74(18.32%)	47(20.43%)	
(-)	330(81.68%)	183(79.57%)	
Ophthalmopathy			
(+)	101(25.00%)	7(3.04%)	
(-)	303(75.00%)	223(96.96%)	

same hospital, with thyroid disease and other autoimmune diseases ruled out. All the subjects, including AITD patients and controls, were Han Chinese and signed the informed consent. The research project was approved by the Ethics Committee of the hospital.

Genotyping

Peripheral venous blood of 2ml from the subjects was collected in an EDTA tube. The genomic DNA was extracted by salting-out method, using RelaxGene Blood DNA System (TIANGEN BIOTECH, BEIJING, China), according to the manufactures' protocol. Genotyping of rs179247, rs12101255, rs2268475, rs1990595 and rs3783938 was performed using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometer (MALDI-TOF-MS) Platform from Sequenom (San Diego, CA, USA).

Clinical phenotype analysis

Correlation analyses between genotypes and clinical manifestations of GD or HT were separately investigated involving 1) the age of onset (≤ 30 years vs. ≥ 31 years [18, 21]); 2) thyroid size ($\leq$ I degree vs. $\geq$ II degree, goiter size is divided into three degrees by palpation: I degree, the goiter can not be seen but could be reached; II degree, the goiter can be seen and reached, but within the sternocleidomastoid; III degree, the goiter exceeds the exterior margin of sternocleidomastoid); 3) presence or absence of AITD family history (defined as the subjects' first-degree relatives including parents, children and siblings or second-degree relatives such as grandparents, uncles and aunts who had AITD occurrence); 4) presence or absence of ophthalmopathy (defined as a distinctive disorder characterized by inflammation and swelling of the extraocular muscles and orbital fat, eyelid retraction, periorbital edema, episcleral vascular injection, conjunctive swelling and proptosis).

Statistical analysis

The clinical data are expressed as $M \pm SD$. All SNPs of the case and control collections were analyzed using HapLoView 4.2 software to perform Hardy-Weinberg equilibrium (HWE) tests, haplotype frequency calculation and linkage disequilibrium (LD) test. LD among these SNPs was measured using the pairwise LD measures D' and r^2 . Haplotype blocks were generated using the default algorithm based on methods established by Gabriel *et al.* [30]. Allele and genotype frequencies between cases and controls were compared with chi-

square test or Fisher's exact test. Differences between groups were determined by the odds ratio (OR) and 95% confidence interval (95% CI), which were calculated according to non-conditional logistic regression model. All statistical analyses were performed using the software SPSS version 13.0. A P value less than 0.05 was considered significant.

Results

Allele and genotyping results

All of these 5 SNPs in both case and control groups were in HWE ($P > 0.05$). Table 2 shows the allele frequencies and case-control association analysis for each SNP. In rs12101255 we found that allele T was significantly more frequent in GD patients than in controls ($P = 0.004$, $OR = 1.408$, $95\%CI = 1.113-1.783$). Likewise, in rs3783938 allele A presented an increased frequency in HT patients compared with controls ($P = 0.025$, $OR = 1.427$, $95\%CI = 1.044-1.950$). rs1990595 observation showed a marginal significance trend between HT subjects and control group ($P = 0.063$). Nevertheless, we did not find any significant difference between cases and controls either in rs179247 or in rs2268475.

Table 3 shows that the TT genotype of rs12101255 was higher in GD patients ($OR = 1.543$, $95\%CI = 1.111-2.143$, $P = 0.009$), which evidently indicated the TT genotype could increase the susceptibility to GD. In addition, the distribution of AA genotype from rs1990595 ($OR = 0.659$, $95\%CI = 0.458-0.948$, $P = 0.024$) and the GG genotype from rs3783938 ($OR = 0.632$, $95\%CI = 0.433-0.922$, $P = 0.017$) were decreased in HT patients compared to the healthy controls.

Haplotype analysis

According to D' value, we detected two LD blocks: rs179247-rs12101255 (within intron1) and rs2268475-rs1990595 (within intron7). Six haplotypes with frequencies greater than 0.05 were identified in our study. The frequency of the AT haplotype in GD patient group was significantly higher than that in control group ($P = 0.01$) with an odds ratio of (1.368), while the GC haplotype was strongly protective ($OR = 0.751$, $P = 0.032$). The haplotypes of block2 were found not to be associated with GD or HT (Table 4).

Genotype and clinical phenotype correlations

Within GD patients, when we compared ophthalmopathy patients with non-ophthalmopathy ones, no associ-

Table 2 Allele and genotype frequencies in AITD patients and controls

SNP	Alleles	Control (%)	GD (%)	<i>P</i>	<i>OR</i>	<i>95%CI</i>	HT (%)	<i>P</i>	<i>OR</i>	<i>95%CI</i>
rs179247	A	328(71.93)	600(76.14)	0.100			311(69.73)	0.468		
	G	128(28.07)	188(23.86)				135(30.27)			
	AA	120(52.63)	230(58.38)	0.261			108(48.43)	0.656		
	AG	88(38.60)	140(35.53)				95(42.60)			
	GG	20(8.77)	24(6.09)				20(8.97)			
rs12101255	C	189(39.37)	255(31.56)	0.004	1.408	1.113-1.783	179(39.08)	0.927		
	T	291(60.63)	553(68.44)				279(60.92)			
	CC	35(14.58)	38(9.40)	0.015	1.446	1.024-2.041	35(15.28)	0.911		
	CT	119(49.59)	179(44.31)				109(47.60)			
	TT	86(35.83)	187(46.29)				85(37.12)			
rs2268475	C	101(21.31)	184(22.77)	0.543			113(24.78)	0.208		
	T	373(78.69)	624(77.23)				343(75.22)			
	CC	13(5.48)	24(5.94)	0.824			15(6.58)	0.434		
	CT	75(31.65)	136(33.66)				83(36.40)			
	TT	149(62.87)	244(60.40)				130(57.02)			
rs1990595	A	344(71.07)	582(72.21)	0.661			301(65.43)	0.063	1.298	
	C	140(28.93)	224(27.79)				159(34.57)			
	AA	125(51.65)	213(52.85)	0.888			95(41.30)	0.074		
	AC	94(38.84)	156(38.71)				111(48.27)			
	CC	23(9.51)	34(8.44)				24(10.43)			
rs3783938	A	90(19.48)	173(21.73)	0.343			116(25.66)	0.025	1.427	1.044-1.950
	G	372(80.52)	623(78.27)				336(74.34)			
	AA	11(4.76)	14(3.52)	0.177			14(6.19)	0.058		
	AG	68(29.44)	145(36.43)				88(38.94)			
	GG	152(65.80)	239(60.05)				124(54.87)			

Table 3 Genotype distributions of rs12101255, rs1990595 and rs3783938 in AITD patients and controls

SNP	Genotype	Control (%)	GD (%)	<i>P</i>	<i>OR</i>	<i>95%CI</i>	HT (%)	<i>P</i>	<i>OR</i>	<i>95%CI</i>
rs12101255	TT	86(35.83)	187(46.29)	0.009	1.543	1.111-2.143	85(37.12)	0.773	1.060	
	TC+CC	154(64.17)	217(53.71)				144(62.88)			
rs1990595	AA	125(51.65)	213(52.85)	0.768	1.050		95(41.30)	0.024	0.659	0.458-0.948
	AC+CC	117(48.35)	190(47.15)				135(58.70)			
rs3783938	GG	152(65.80)	239(60.05)	0.152	0.780		124(54.87)	0.017	0.632	0.433-0.922
	GA+AA	79(34.20)	159(39.95)				102(45.13)			

Table 4 Haplotype analysis in AITD patients and controls

Haplotype	Control (Frequency)	GD (Frequency)	<i>P</i>	<i>OR</i>	<i>95%CI</i>	HT (Frequency)	<i>P</i>	<i>OR</i>	<i>95%CI</i>
Block 1									
AT	275(0.606)	534(0.678)	0.010	1.368	1.076-1.740	268(0.600)	0.882		
GC	131(0.289)	184(0.234)	0.032	0.751	0.578-0.976	135(0.302)	0.642		
AC	48(0.105)	64(0.081)	0.146			39(0.088)	0.353		
Block 2									
TA	337(0.710)	581(0.721)	0.705			300(0.657)	0.082		
CC	101(0.213)	184(0.228)	0.528			113(0.248)	0.208		
TC	37(0.078)	41(0.051)	0.050			43(0.095)	0.377		

Table 5 The allele frequencies of rs179247 and rs12101255 in ophthalmopathy and non-ophthalmopathy patients and controls

SNP	Alleles	Control n(%)	Ophthalmopathy				Non-ophthalmopathy			
			n(%)	<i>P</i>	<i>OR</i>	95% <i>CI</i>	n(%)	<i>P</i>	<i>OR</i>	95% <i>CI</i>
rs179247	A	328(71.93)	157(80.10)	0.028	1.571	1.047-2.357	443(74.83)	0.291		
	G	128(28.07)	39(19.90)				149(25.17)			
rs12101255	C	189(39.38)	60(29.70)	0.017	1.537	1.080-2.188	195(32.18)	0.014	1.369	1.066-1.758
	T	291(60.62)	142(70.30)				411(67.82)			

ation was found. When we compared GD ophthalmopathy patients with healthy controls, significant differences were observed. The most obvious disparity was that allele A from rs179247 was increased in patients with ophthalmopathy ($P=0.028$, $OR=1.571$, $95\%CI=1.047-2.357$). Besides, allele T from rs12101255 was significantly higher in both ophthalmopathy and non-ophthalmopathy groups in comparison with controls, which was parallel with the above results (Table 5). No relationships were found between the other clinical phenotypes and these SNPs (data not shown).

Discussion

TSHR gene is located on chromosome 14q31, containing 10 exons and 9 introns, which encode a 764 amino acid protein composed of extracellular, transmembrane and intramembrane areas [31]. TSHR is sometimes cleaved into distinct A and B subunits at or near the cell surface [22]. The A-subunit belongs to the extracellular domain, which includes 394 amino acids encoded by exons 1-9. The B-subunit represents the transmembrane region, comprising 349 residues encoded by exon 10. TSHR belongs to G-protein coupled receptors family, acting *via* binding with its ligand G-protein mediating roles, exerts corresponding biological effects through cAMP or diphosinositide pathway. The cAMP pathway can stimulate thyroid epithelial cells growth, up-regulate the synthesis of thyroid hormone or other autoantigens-TG and TPO. However, diphosinositide pathway can stimulate protein iodination and promote thyroid hormone production. Therefore, it is suggested that TSHR abnormal alterations could cause AITD through the above-mentioned cascade reactions.

The present study investigated the association between five intronic SNPs of TSHR and GD or HT in a Chinese Han population, and correlations have been observed. Our study for single SNPs had 80% power ($\alpha=0.05$) to detect a significant difference between

cases and controls. Regarding the allele frequencies and genotype analysis of intron 1, we found that rs12101255 major allele T was significantly increased in GD cohorts and raised the risk of GD by 40.8%. Besides, the TT genotype frequencies in GD and control collections were 46.29% and 35.83%, respectively. Therefore, it is clear that the TT could increase the risk of GD. Although no significant differences were observed in the allele and genotype distribution of rs179247 between case and control subjects, the major allele A was highly increased in ophthalmopathy patients with GD compared with healthy controls. In other words, the A of rs179247 conferred risk of ophthalmopathy in GD patients by 57.1%. Moreover, in the haplotype analysis, the AT haplotype from block1 (rs179247, rs12101255) was significantly different between GD and control groups and increased the risk of GD by 36.8%. Conversely, the GC haplotype was decreased in GD patients, which reduced the risk of suffering from GD. According to another case-control cohort Chinese study, which involved 199 GD patients and 208 control subjects, rs179247 and rs12101255 are not involved in the pathogenesis of GD [32]. The difference may be attributed to its small sample size. Similarly our findings disaccorded with the TSHR intron1 SNPs study in three independent European cohorts [14] mainly due to its inadequate sample size. Many researches demonstrate that haplotype contains genetic information of multiple SNPs; therefore, using haplotype would generate significantly better effects than single SNP in the complex characters analysis. Consequently these effects should also be replicated in larger Chinese cohorts and other ethnic populations.

The minor allele A of rs3783938 within intron8 was significantly increased in HT patients compared with that in controls, and thus resulted in an increased risk of HT by 42.7%. What biological role that the allele A may play is unclear. It is possible that the associated SNP include functional variant, or this SNP may contribute to autoantibody production or other immu-

nological derangement in HT. The AA genotype frequency was 6.19% and 4.76%, respectively, in HT and control groups. Although it showed slight elevation in HT patients, no significant difference was found. Neither rs2268475 nor rs1990595 within intron7 was observed to be significantly associated with GD or HT in allele, genotype or haplotype analysis. In our research we found none of the three SNPs in intron7 and 8 was associated with GD pathogenesis. Our findings were different from those on the Japanese populations [12], which may attribute to ethnic diversity and other different elements.

So far, the pathogenesis of AITD has not been fully elucidated. More recently it has been suggested that the TSHR protein can undergo a post-translation intra-molecular cleavage event resulting in shedding of the TSHR A-subunit, which is a substantial auto-antigen [22]. The truncated mRNA transcripts ST4 and ST5 encode the majority of soluble A-subunit directly, thus increas-

ing the chances of autoantibody production against the TSHR. It has been reported that rs179247 and rs12101255 increase the level of ST4 and ST5 expressions compared to full length TSHR, and may support the hypothesis for disease pathogenesis [13].

In summary, we can conclude that the SNPs in TSHR intron1 and 8 are significantly associated with pathogenesis of AITD. In order to capture the AITD mechanism conferred by TSHR, further genetic studies combined with expression data and functional researches will be needed to validate, and thus succeed in finding novel therapeutic targets.

Acknowledgement

We thank the research participants who took part in the studies described in this report. This work was supported by grants from the National Natural Science Foundation of China (30871184, 81070627).

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