Endocr (2007) 32:170–174
DOI 10.1007/s12020-007-9027-9

ORIGINAL PAPER

Lack of association between interleukin-4 gene polymorphisms and autoimmune thyroid diseases amongst Taiwanese Chinese

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Received: 5 October 2007 / Accepted: 5 November 2007 / Published online: 5 December 2007

Abstract Graves' disease (GD) and Hashimoto's thyroiditis (HT) are both common autoimmune diseases of the thyroid gland (AITD). The IL-4 is involved in both humoral and cellular immunity. The aim of this study was to test whether the IL-4 gene could be used as a genetic marker to predict the development of AITD amongst the Chinese population of Taiwan. For this study, a normal control group of 105 healthy subjects and two experimental groups featuring individuals afflicted with either GD (104 patients) or HT (109 patients) were examined. Polymerase chain reaction (PCR) was used to analyze the variable number of tandem repeats (VNTRs) polymorphism for the IL-4 gene intron 3 and PCR-based restriction analysis using endonuclease *Bsm*FI was undertaken for the same gene at the promoter −590 position. We found no significant difference in the frequencies of presence of genotype and allelic variants for the IL-4 gene at both the intron 3 and the promoter regions between the normal control group and each of the two patient groups. These findings suggest that the IL-4 gene polymorphisms that arise at either intron 3 or promoter −590 positions are not suitable genetic markers for AITD among Taiwanese Chinese.

Keywords Graves' disease · Hashimoto's thyroiditis · Interleukin-4 · Intron · Promoter · Polymorphism

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Introduction

Graves' disease (GD) and Hashimoto's thyroiditis (HT) are both common autoimmune diseases of the thyroid gland (AITD). Clinically, active GD patients virtually always appear hyperthyroid, whereas HT patients may be euthyroid, hypothyroid, or occasionally, hyperthyroid. Although the histopathological appearance of both disorders are different in some ways, the thyroid glands of patients suffering from each disorder all present with a rather diffuse cellular infiltration, which is composed of T- and B-cells and other inflammatory cells (especially macrophages), this suggesting the contribution of cell-mediated immunity, as well as humoral immunity, to the pathogenesis of these two diseases [1–3]. In practice, an abundance of antibodies (Abs) against certain thyroid-specific components, such as thyrotropin receptor, microsome/thyroid peroxidase and thyroglobulin, can be measured from the sera of the majority of individuals afflicted with either GD or HT [4, 5].

The pathogenesis of GD and HT involves mainly the respective actions of thyrotropin receptor Ab (TRAb) and anti-microsomal Ab (AMiA), which is now usually referred to as anti-thyroid peroxide (anti-TPO) Ab, upon the thyrocytes [4–6]. Both TRAb and AMiA are IgG_1 Abs. The formation of intrathyroidal Abs is a complicated process of immunological reactions. Upon stimulation by various

exogenous and endogenous factors, intrathyroidal $CD4^+$ helper lymphocytes (Th) differentiate into Th1 and Th2 subtypes which feature different cytokine production [7]. Th2 cells produce interleukin (IL)-4 and various other cytokines, and provide optimal help for humoral immunity, which is responsible for the production of certain Abs [8]. From animal and human studies, IL-4 has been observed to exert effects relating to the growth and proliferation of B-cells and to the production of serum IgG_1 [9–11]. Further, IL-4 may also enhance the proliferation of certain precursors of cytotoxic T cells (CTLs) and their subsequent differentiation into active CTLs, two events which also play a critical role in the development of both GD and HT [12, 13].

To the best of our knowledge, the underlying mechanisms that trigger the immunological reactions in AITD are still quite unclear and are believed by many to be influenced by multiple genetic and environmental factors. The genetic mechanism underlying AITD would appear to be quite complex, and to the best of our knowledge, most studies that have attempted to deal with this mechanism have focused upon the HLA region and the cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*) [14–16]. It would appear, however, that other genes are also involved in the inheritance of AITD. IL-4 gene polymorphisms were observed to have been associated with an individual's susceptibility to GD in a study for a Caucasian population in the United Kingdom (UK) [17], although for another study, also undertaken in the UK, similar results were not obtained [18]. With regard to HT, to the best of our knowledge, there still appears to be nothing published in the literature the authors of which have claimed to have demonstrated an association between such polymorphisms and HT development. To further test whether an IL-4 gene polymorphism could constitute a marker of susceptibility to AITD among Taiwanese Chinese individuals, we screened two polymorphic regions of the IL-4 gene (promoter −590 and intron 3 regions), using polymerase chain reaction (PCR) analysis, in order to compare these polymorphic regions for either GD or HT patients with corresponding regions for normal controls deriving from the general Taiwanese population.

Results

From the initial examination for genotype frequencies, the IL-4 gene polymorphisms at both promoter −590 and intron 3 regions were found to be in HWE for both control and patient groups.

For the analyses conducted for GD patients, no statistically significant difference in distribution of genotype frequencies for the IL-4 gene at both promoter −590 and intron 3 regions was detected (respectively, $P = 0.636$ and $P = 0.642$; Fisher's exact test; Table 1) as compared with the corresponding results for normal controls. The difference in distribution of allelic frequencies for the IL-4 gene at both promoter −590 and intron 3 sites (respectively, $P = 0.962$ and $P = 0.648$; χ^2 test; Table 1) between the GD patient and control groups were also not statistically significant.

With regard to the analyses for HT patients, the results were similar to those for GD patients. For the distribution of genotype frequencies, no statistically significant difference for the IL-4 gene at both the promoter −590 and intron 3 regions (respectively, $P = 0.682$ and $P = 0.520$; Fisher's exact test; Table 2) were detected between patient and control groups. For the distribution of allelic frequencies, the difference at both these sites (respectively, $P = 0.954$ and $P = 0.365$; χ^2 test; Table 2) between the HT and control groups also did not prove to be statistically significant.

Further subgroup analyses were performed, and no significant association between the IL-4 gene polymorphisms at both the promoter −590 and intron 3 regions and either TRAb titer (≥ 30% or < 30%) or exophthalmos (presence or absence) among GD patients was detected. There was also no significant difference among the IL-4 gene polymorphisms at these two sites and either AMA titer (≥ or < 1:1,600) or TSH level (≥ or < 5 mU/l) among HT patient group (data not shown).

Table 1 Distribution of genotype and allelic frequencies for the IL-4 gene promoter −590 and intron 3 comparison between GD patients and normal controls

	GD patients $n = 104$ (%)	Normal controls $n = 105$ (%)	P
IL-4 promoter −590			
Genotype			0.636[a]
TT	68 (65.4%)	67 (63.8%)	
TC	32 (30.8%)	36 (34.3%)	
CC	4 (3.8%)	2 (1.9%)	
Allelic variant			0.962[b]
T	168 (80.8%)	170 (81.0%)	
C	40 (19.2%)	40 (19.0%)	
IL-4 intron 3			
Genotype			0.642[a]
RP1/RP1	74 (71.2%)	80 (76.2%)	
RP1/RP2	28 (26.9%)	21 (20.0%)	
RP2/RP2	2 (1.9%)	4 (3.8%)	
Allelic variant			0.648[b]
RP1	176 (84.6%)	181 (86.2%)	
RP2	32 (15.4%)	29 (13.8%)	

[a] Fisher's exact test

[b] χ^2 test

Table 2 Distribution of genotype and allelic frequencies for the IL-4 gene promoter −590 and intron 3 comparison between HT patients and normal controls

	HT patients n = 109 (%)	Normal controls n = 105 (%)	P
IL-4 promoter −590			
Genotype			0.682[a]
TT	71 (65.1%)	67 (63.8%)	
TC	34 (31.2%)	36 (34.3%)	
CC	4 (3.7%)	2 (1.9%)	
Allelic variant			0.954[b]
T	176 (80.7%)	170 (81.0%)	
C	42 (19.3%)	40 (19.0%)	
IL-4 intron 3			
Genotype			0.520[a]
RP1/RP1	76 (69.7%)	80 (76.2%)	
RP1/RP2	29 (26.6%)	21 (20.0%)	
RP2/RP2	4 (3.7%)	4 (3.8%)	
Allelic variant			0.365[b]
RP1	181 (83.0%)	181 (86.2%)	
RP2	37 (17.0%)	29 (13.8%)	

[a] Fisher's exact test

[b] χ^2 test

Discussion

The study conducted by Hunt et al. [17] suggested that the IL-4 gene polymorphism was associated with an involved individual's susceptibility to GD in a UK population. Our present study, however, does not find any association between IL-4 gene polymorphisms and an individual's susceptibility to GD amongst Taiwanese Chinese. Further, we also fail to observe any relationship between such gene polymorphisms and HT development. Our data demonstrate that the distribution of both genotype and allelic frequencies for the IL-4 gene, at either promoter −590 or intron 3 regions, amongst a pool of AITD patients are similar to the corresponding distribution for normal healthy individuals. In addition, the IL-4 gene polymorphisms also do not influence the clinical status of AITD.

The IL-4 gene is located on the long arm of chromosome 5 (5q23.3–31.2) [19]. The major polymorphisms of the IL-4 gene appear at the promoter −590 sate and feature a T/C polymorphism, and at intron 3 revealing a 70-bp variable number of tandem repeats (VNTRs) (RP1 and RP2) polymorphism [20, 21]. In theory, the polymorphism arising at the promoter region may interfere with normal gene expression and the resultant development of a clinical immune disorder. In actuality, the IL-4 gene promoter −590 polymorphism has been observed to be associated with asthma and atopic dermatitis [22, 23]. Previous studies investigating the relationship between such polymorphism and GD susceptibility, however, would appear to be limited in number and the results somewhat conflicting [17, 18]. Although Hunt and coworkers observed that the T allelic variant in this region may confer modest protection against the development of GD, the study by Heward and colleagues, also conducted in the UK, did not reveal any difference in genotype and allelic frequencies between patients and normal controls [17, 18]. The data deriving from our study also revealed no apparent association between polymorphism at this site and AITD susceptibility. The discrepancy in results deriving from different studies may be due to different sample sizes, population stratification and/or statistical artifacts, however, the different genetic pools represented in the different racial populations investigated may be another relevant factor.

With regard to the IL-4 gene intron 3, the function of the RP1/RP2 polymorphism is still unknown; it may possibly be that distinct numbers of VNTRs copies might affect the transcriptional activity of the IL-4 gene [24]. This IL-4 gene polymorphism at intron 3 has been reported to be associated with rheumatoid arthritis and immune thrombocytopenic purpura [24, 25]. To the best of our knowledge, however, there exist no published studies in the literature the authors of which claim any association between such polymorphism and the development of AITD. Our present study also fails to substantiate the function of the IL-4 gene intron 3 polymorphism as regards an involved individual's susceptibility to either GD or HT. Further study is thus necessary to identify the function of the IL-4 gene intron 3 polymorphism.

Cytokines constitute a large group of protein hormones, a significant proportion of the members in this group contributing to the development of various immune disorders. Although our present study fails to demonstrate any association between IL-4 gene polymorphisms and AITD susceptibility, it would still appear worthwhile for future researchers to investigate other cytokine-gene polymorphisms in order to attempt to elucidate an individual's genetic susceptibility to AITD.

Materials and methods

Patient selection

A total of 104 unrelated Chinese GD patients (83 women) aged between 17 years and 71 years (mean 35.6 ± 12.7) and 109 HT patients (98 women) aged between 17 years and 69 years (mean 35.9 ± 12.3) were enrolled into this study over the period of from January 2003 to January 2005 inclusively. All study-participating patients were of the Han race and resided in mid-Taiwan. None of the

female patients were pregnant at the time of the study, and none had delivered a baby/babies within the entire 1-year period prior to study enrolment. The presence of hyperthyroidism, a diffuse goiter and a positive serum TRAb titer (>10%), supported by infiltrative ophthalmopathy and a positive serum AMiA and/or antithyroglobulin Ab (ATA) titer, were used to define GD. With respect to the HT sufferers in our study group, none of these patients revealed any current or previous history of hyperthyroidism and/or thyroid-associated ophthalmopathy. The presence of a palpable goiter and high serum AMiA and/or ATA titer(s) (1:100 or greater), with a negative TRAb titer, were used as the definitive criteria to specifically define HT. The ultrasonographic examination of thyroid gland revealed a diffuse hypoechogenic pattern and no evidence of nodular lesions for both groups of patients. The control group consisted of 105 (92 women) ethnically and residentially matched healthy volunteers over the age of 40 years who featured neither goiter nor any evidence of thyroid dysfunction. None of these controls revealed the presence of any positive antithyroid Abs in their sera. Further, these individuals also exhibited no previous personal or family history of thyroid disease or of any autoimmune disease. This study was approved by the institutional ethics committee of our institution, and informed consent was requested of, and obtained from, each study subject prior to their enrolment in this study.

Polymerase chain reaction

Experimental genomic DNA was prepared from peripheral blood using a genomic DNA-isolation reagent kit (Blossom, Taipei, Taiwan). Approximately 50 ng of genomic DNA was mixed with 20 pmol of each PCR primer in a total volume of 25 μl containing 10 mM Tris–HCl, pH = 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, and one unit of AmpliTaq DNA polymerase (PerkinElmer, Foster City, CA, USA). The primers were respectively designed as sense 5'-AGGCTGAAAGGGGGAAAGC-3' and antisense 5'-GT GTTCACCTCAACTGCTCC-3' for IL-4 gene intron 3, and 5'-ACTAGGCCTCACCTGATACG-3' and 5'-GTT GTAATGCAGTCCTCCTG-3' for IL-4 gene promoter −590, as described by Mout et al. and Walley et al. [20, 21]. PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (PerkinElmer). Cycling conditions were set as follows: 35 cycles at 92°C for 30 s, 58°C for 30 s and 72°C for 30 s for both polymorphisms.

For IL-4 gene intron 3, 10 μl of PCR amplification product was loaded onto a 3% agarose gel containing ethidium bromide for electrophoresis, and each allele was recognized according to its size. The 70 base-pair (bp) length of VNTRs of the polymorphism for IL-4 gene intron 3 were classified as RP1 (183 bp) and RP2 (253 bp). For polymorphism at the IL-4 gene promoter region, the PCR amplification product was further analyzed by means of a restriction fragment length polymorphism (RFLP) method using *Bsm*FI (New England Biolabs, Beverly, CA, USA) digestion. The C allele at position −590 showed up as 192-bp and 60-bp fragments upon agarose electrophoresis. The T allele was 252 bp in length, and was encoded at position −590. Thus, the genotypes at this site were classified into excisable allelic homozygote (CC) and non-excisable allelic homozygote (TT), and heterozygote (CT). The genotype analyses of patients and controls were performed contemporaneously in the same laboratory and the gels were inspected by investigators who were blinded to the clinical phenotypes of the individuals being studied. If any doubtful or unclear readings for genotype arose, the associated PCR procedure was repeated, in order to determine the exact genotype for each individual for whom a suspect result had arisen previously.

Statistical analysis

The genotype and allelic frequencies of the IL-4 gene polymorphisms for the GD and HT patient groups were separately compared statistically with the corresponding polymorphisms for the control group using the χ^2 test performed with the Statistical Package for Social Sciences (SPSS) Version 8.01 software (SPSS for Windows, SPSS Inc.; Chicago, IL, USA) following the examination of allelic frequencies incorporating the application of Hardy-Weinberg equilibrium (HWE) test for genotype frequencies. When the assumption of the χ^2 test was violated (i.e., when one cell had an expected count of <1, or <20% of the cells had an expected count of <5), Fisher's exact test was used. Results were considered to have achieved statistical significance when the probability of findings occurring simply by chance was less than 5% ($P < 0.05$).

Acknowledgment This study was supported by a grant from the China Medical University Hospital (DMR-96-017).

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