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ORIGINAL PAPER

Intercellular adhesion molecule 1 gene polymorphisms do not contribute to Graves' disease in Chinese patients

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Abstract
Objective In order to study the association of G241R polymorphism of ICAM-1 gene with an earlier onset of Graves' disease (GD) and the susceptibility of K469E polymorphism to Graves' ophthalmopathy (GO) in Chinese population.
Study Design A case-control and replication study was performed in 212 GD patients and 204 healthy subjects to analyze the genotypes. Furthermore the distribution of ICAM-1 genotypes was investigated in subgroups of patients with GD according to the onset age and the ophthalmopathy.
Results No G241R polymorphism of ICAM-1 gene was detected in Chinese. No significant differences of allele and genotype frequencies regarding K469E polymorphism were found between GD patients and healthy controls ($\chi 2$ = 0.092, P = 0.762; $\chi 2$ = 1.089, P = 0.580). In addition, the genotype–phenotype correlation was not identified either.
Conclusions We found no association of G241R and K469E polymorphisms of the ICAM-1gene with the development of GD in a Chinese population. However, we could not rule out possible contributions of other polymorphisms of the ICAM-1gene to the pathogenesis of GD. Therefore, further studies are needed to elucidate the role of ICAM-1gene in Graves' disease in different population.

Keywords ICAM-1 · Thyroid · Graves' disease · Graves' ophthalmopathy · Polymorphism

Introduction

Graves' disease (GD) is an autoimmune disorder frequently leading to varying degrees of hyperthyroidism and ophthalmopathy [1]. The nature of autoimmune responses in the thyroid and the orbit which are associated with GD and Graves' ophthalmopathy (GO), respectively, have not been well elucidated, although TSH receptor was considered as a crucial autoantigen in GD patients [2, 3]. In general, both environmental and genetic factors are involved in GD and GO development [4–6]. A polygenic susceptibility has been reported including major histocompatibility complex [7, 8], cytotoxic T lymphocyte antigen-4 [9 –11], interferon-α [12, 13], TNF-β [14] , PTPN22 [15, 16], thyrotropin receptor (TSHR) [17], CD40 [18–20], and interleukin (IL)-13 genes [21]. However, two single nucleotide polymorphisms of the intercellular adhesion molecule 1 (ICAM-1) gene was reported to be associated with GO and possibly to influence the age of GD onset [22].

ICAM-1, a ligand bound to β-2 integrins in leucocytes has been known to participate in the migration and activation

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of lymphocytes, monocytes, and neutrophils, which indicates an association with autoimmune diseases [23–25]. Human ICAM-1 gene is located on chromosome 19 with a single-copy and carries two polymorphic sites at codon 241 at exon 4 (G241R; Gly/Arg) and codon 469 at exon 6 (K469E; Lys/Glu) respectively. In the present study, we investigated the association of ICAM-1polymorphism with GD and some other phenotypes in Chinese population-based on the genetic heterogeneity of GD [6, 26, 27].

Subjects and methods

Patients and controls

The study was carried out in 212 unrelated Chinese patients (female/male ratio, 3.6:1) with GD: 127 subjects with disease onset less than 40 year of age (range, 8–39 year; mean, 27.6 ± 7.7 year) and 85 subjects with the onset at 40 year of age or older (40–81 year; mean age, 48.5 ± 6.7 year). Patients with GD were sequentially recruited from the Shanghai Clinical Center for Endocrine and Metabolic Diseases in Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine between April 2005 and February 2006. GD was diagnosed on the basis of clinical observation (diffuse goiter), biochemical criteria of thyrotoxicosis (TSH <0.05 mIU/l and increased free T3 and/or free T4), and the presence of TSH receptor antibodies [28]. Thyroid eye disease was diagnosed by experienced ophthalmologists and was classified using the NOSPECS classification [29]. For the statistical analysis, patients with classes 2–6 were considered as having GO.204 healthy Chinese volunteers (female/male ratio, 4:1) from the same region of China without family history of GD or other autoimmune diseases served as the control group. Informed consent was obtained from every participant and the study was approved by the ethics committee of the Ruijin Hospital affiliated to Shanghai JiaoTong University School of Medicine.

Genotype analysis

Genomic DNA from peripheral blood was extracted using a commercially available kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The polymorphism G241R was detected by direct sequencing. A 474 base-pair (bp) fragment containing the polymorphic site (G241R) was amplified using ICAM-1specific primers (forward: 5′-CGTCCATCCCTGTCTGCTC-3′; reverse: 5′-ACT GTCACCTCGGTCCCTTC-3′). The PCR was performed in a volume of 50 μl containing 1.5 mM $MgCl_2$, 10 mM dNTP, 50ng genomic DNA, 20 μmol of each primer and 2.5 UTaq DNA polymerase (Sangon, Shanghai, China). Amplification was performed with preheating at 95°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s for 30 cycles. The PCR products were purified using the QIA quick PCR purification kit(QIAGEN, Strasse, Germany) and sequenced in both sense and antisense directions on CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc,Fullerton, CA, USA). A 712-bp PCR fragment covering the K469E polymorphism at exon 6 was generated using primers (forward: 5′-CAAGGTGACGCTGAATGG -3′; reverse: 5′-CGGTTA TAGAGGTACGTGCTG-3′). The PCR amplification procedure was described as above. The polymorphism K469E was detected by CEQ SNP Detection system: 6 μl of the PCR products was incubated with 2 U of shrimp alkaline phosphatase (SAP, USB Corp.Ohio USA) and 0.1 U exonuclease I (ExoI, USB Corp.,Ohio USA) at 37°C for 1 h, followed by an enzymatic inactivation step of 75°C for 15 min. Primer extension (minisequencing) reactions were carried out in a volume of 10 μl, containing 0.5 μl of premixed sequenase and fluorescently labeled dideoxnucleotides from a CEQ SNP Primer Extension Kit (Beckman Coulter), 1 μl of purified PCR product, 100 nM of primer and 0.6× PCR buffer (6 mM TRIS–HCl, 0.9 mM MgCl2, 30 mM KCl and 0.06% Triton X-100). The cycling profiles were 96°C for 10 s, 50°C for 10 s and 72°C for 30 s for 30 cycles. About 2.5 μl of labeled primers were cleaned up by incubation with 1 U of SAP at 37°C for 1 h, followed by an inactivation step of 75°C for 15 min. 1 μl of sample was run on a CEQ™ 8800 Genetic Analysis System according to the manufacturer's instruction.

Statistical analysis

The differences in the distribution of alleles and genotypes among the studied groups were estimated by χ^2 test for 2×2 or 2×3 tables (SPSS software version 11.5 SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$. Deviation from the Hardy-Weinberg equilibrium was tested using the Pearson's χ^2 test statistic for any of the SNPs under consideration.

Results

Genotypes and allele frequencies of G241R polymorphisms

G241R polymorphism could not found in 100 healthy subjects, which was in accordance with the data provided by International Hapmap Project. In order to further confirm the negative G241R polymorphism in GD population, we performed pool sequencing using DNA samples from 50 GD patients. The G241R polymorphism could not be detected either.

Genotypes and allele frequencies of K469E polymorphisms

The allele frequencies and genotype distribution of the ICAM-1gene polymorphism between GD patients and control subjects are shown in Table 1. The genotype distributions are all compatible with the Hardy–Weinberg law. However, we did not find a significant difference for the frequencies of alleles or genotypes between the GD patients and the control. The allele A frequency is much higher in patients with GD than the control, however a statistical significant difference could not be detected either ($\chi^2 = 0.092$, $P = 0.762$).

The significant differences of genotype and allele frequencies were not detected among the patients with evident ophthalmopathy (NOSPECS class 2 or more) and those with no or mild ophthalmopathy (NOSPECS class 0–2) (Table 1). Regarding the onset age, patients with an earlier onset (<40 year of age) had a higher frequency of allele A compared with the healthy controls. However, the difference did not reach statistical significance ($\chi^2 = 1.107$, $P = 0.575$).

Power calculation

In order to estimate the risk of acquiring false-negative results due to the small sample size, we used QUANTO software (http://www.hydra.usc.edu/gxe) to calculate the power of association studies. At the 0.05 level of significance with the two-sided test for the K469E polymorphism, the present study has more than 93% power to detect an effect at a relative risk of 2.0 in the groups of GD patients and healthy controls, 69% power in the subgroup of GD patients with and without ophthalmopathy (GO), and 68% power in the subgroup of GD patients with an earlier onset of GD and the subjects with a later onset, under a recessive genetic model.

Discussion

In this present study, we did not find a significant difference in the frequencies of alleles or genotypes between the GD patients and healthy controls which is in accordance with a report in Polish GD patients [22]. Kretowskia et al. also reported that the G241R polymorphism at exon 4 was associated with an earlier onset of GD and the K469E polymorphism at exon 6 could be predisposed to GO. However, with stratification of onset age, we found no differences for the distribution of K469E and G241R alleles frequencies between patients with a onset age of less than 40 year and those with a later onset or healthy subjects. Moreover, we did not find the differences of the allele frequencies of G241R and K469E in GO patients compared with the subjects without GO or the healthy subjects, which are not in accordance with Kretowskia's report.

The distribution of ICAM-1 polymorphisms appears to vary in different racial population. We did not find a G241R polymorphism in Chinese population, which was already reported in European populations, including Polish,

Table 1 Genotypes and allele frequencies of K469E of ICAM-1gene polymorphisms in GD and controls

	GD onset		GD ophthalmopthy		All GD	Controls
	<40 year ($n = 127$)	≥40 year ($n = 85$)	With ($n = 101$)	Without ($n = 111$)	($n = 212$)	($n = 204$)
Frequency of alleles						
A	178[bc](70.1)	114(67.1)	137[de](67.8)	155(69.8)	292[a](68.9)	277(67.9)
G	76(29.9)	56(32.9)	65(32.2)	67(30.1)	132(31.1)	131(32.1)
Frequency of genotypes						
GG	14[bc](11.0)	11(12.9)	14[de](13.9)	11(9.9)	25[a](11.8)	21(10.3)
AG	48(37.8)	34(40.0)	37(36.6)	45(40.5)	82(38.7)	89(43.6)
AA	65(51.2)	40(47.1)	50(49.5)	55(49.5)	105(49.5)	94(46.1)

Values given are the number of alleles and genotypes, with the percentage in *parentheses*

[a] The difference in the frequencies of alleles and genotypes between all GD patients and controls was not statistically significant($\chi^2 = 0.092$, $P = 0.762$; $\chi^2 = 1.089$,$P = 0.580$)

[b] The difference in the frequencies of alleles and genotypes among subjects with the onset of GD at less than 40 year compared with subjects with a later onset was not statistically significant ($\chi^2 = 0.433$, $P = 0.510$; $\chi^2 = 0.397$,$P = 0.820$)

[c] The difference in the frequencies of alleles and genotypes among subjects with the onset of GD at less than 40 year compared with controls was not statistically significant ($\chi^2 = 1.107$, $P = 0.575$; $\chi^2 = 1.107$,$P = 0.575$)

[d] The difference in the frequencies of alleles and genotypes among GD patients with ophthalmopathy and the controls was not statistically significant t($\chi^2 = 0.000$, $P = 0.986$; $\chi^2 = 1.717$,$P = 0.424$)

[e] The difference in the frequencies of alleles and genotypes among GD patients with or without opththalmopathy was not statistically significant (χ^2=0.197, P=0.657; χ^2=0.909,P=0.635)

British and Italian [22, 30, 31]. Interestingly, this G241R polymorphism was not detected in another Asian population, Japanese either [32]. In addition, the distribution of K469E polymorphism in healthy Chinese (0.68/0.32) was also different from that in British Caucasians (0.56/0.44, $P<0.05$), Finnish (0.52/0.48, $P<0.0001$) and Spanish people (0.50/0.50, $P<0.0001$) [30, 33]. Therefore, the dissimilar genetic and environmental backgrounds may result in the different outcomes. This is an important issue because disease susceptibility in multifactorial disorders is dependent on both the prevalence of the polymorphism in the population and exposure to environmental factors [34]. Given that susceptibility genes may have different effects in ethnically distinct populations and/or varying effects depending on allele frequencies [35], it is possible that this polymorphism is associated with GD in Polish patients, but not in those from other ethnic groups, including the Chinese population. Interestingly, vitamin D receptor gene polymorphism was also associated with GD only in some ethnic populations [36].

It was reported that ICAM-1 played an important role in the process of lymphocytes attached to the cultured Graves' thyroid cells [37]. The previous studies had demonstrated that serum ICAM-1 was increased in patients with untreated GD as compared with the healthy controls [38, 39]. Hara et al. observed an enhanced activity of ICAM-1 in adults with hyperthyroidism [40]. However, we could not find the consistent evidence at genetic levels in the present study. We should have been able to detect the association of K469E polymorphism of ICAM-1 gene with GD if it did exist in Chinese population, since the present analysis had a greater than 90% power to detect a disease association assuming an odds ratio of 2.0.

In conclusion, we found no association of G241R and K469E polymorphisms of the ICAM-1gene with the development of GD in a Chinese population, which contradicted with that observed in Poland Caucasian. However, we could not rule out possible contributions of other polymorphisms of the ICAM-1gene to the pathogenesis of GD. Therefore, further studies are needed to elucidate the role of ICAM-1gene in Graves' disease.

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