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No major impact of insertion/deletion polymorphism of the angiotensin-converting enzyme gene on thyroid-associated ophthalmopathy

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Aim: To investigate the association between ACE gene I/D polymorphism and thyroid-associated ophthalmopathy (TO) in the Turkish population.

Materials and methods: A total of 105 patients with TO and 102 healthy control subjects were enrolled in this case-control study. Genomic DNA was extracted from peripheral blood leukocytes, and I/D polymorphism of the ACE gene (*ACE*) was determined using a polymerase chain reaction.

Results: There were no marked changes in allelic frequencies and genotype distribution of *ACE* I/D polymorphism between patients and control subjects. The distribution of I/D polymorphism and allele frequencies of TO patients were not significantly different from those of the controls: DD genotype 34.3% vs. 41.2%; ID genotype 46.7% vs. 43.1%; and II genotype 19.0% vs. 15.7%; D allele 57.6% vs. 62.7%; I allele 42.4% vs. 37.3% ($P > 0.05$). Genotype and allele frequencies for men and women were also similar between the groups.

Conclusion: Our data suggest that ACE gene I/D polymorphism does not constitute a risk factor for TO in the Turkish population. Further studies using greater numbers of patients from different populations are required to clarify the role of the ACE gene in conferring susceptibility to TO.

Key words: ACE gene, allele frequency, I/D genotype, polymorphism, thyroid-associated ophthalmopathy

1. Introduction

Thyroid-associated ophthalmopathy (TO), also called ophthalmic Graves' disease, Graves' ophthalmopathy, Graves' orbitopathy, or thyroid eye disease, is considered an autoimmune disease based on a heterogeneous genetic disorder, but despite extensive research performed on the topic, its causative effects are still not fully understood. TO patients are not strictly hyperthyroid, but a minority of patients (around 10% or less) are euthyroid or hypothyroid (1). As for other autoimmune diseases, a number of environmental and genetic factors are thought to be involved in the pathogenesis of TO (2). Among environmental factors, iodine, drugs (e.g., amiodarone), stress, and smoking are known to play roles in the pathogenesis of TO. TO is seen most frequently in relatives of TO patients. It is documented that candidate genes including human leukocyte antigen (HLA), cytotoxic T-lymphocyte antigen-4 (CTLA-4), tumor necrosis factor-a (TNF-a), interferon-g (IFN-g), intercellular adhesion molecule-1 (ICAM-1), and thyroid-stimulating hormone receptor (TSHR) play roles in the pathogenesis of TO (3). In orbital soft tissue, TO-induced chronic inflammation leads to edema, glucosaminoglycan synthesis, proliferation of fibroblasts and adipocytes (early phase), and fibrosis (late phase). These events can then cause sight-altering phenomena (e.g., elevation of orbital pressure, proptosis, dry eye syndrome, exposure keratopathy, optic neuropathy, fibrotic lid retraction, forward displacement of the eyeball, and diplopia) to occur (4). The incidence of TO has been reported to vary from 30% to 60% among races (2,5). TO occurs in about 40%–50% of cases of Graves' disease, and is sight-threatening (because of optic neuropathy or corneal ulceration) in 3%–5% (5).

Angiotensin I-converting enzyme (ACE) is a carboxypeptidase that plays a central role in the renin–angiotensin system (RAS) by catalyzing the conversion

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of angiotensin I to vasoactive angiotensin II (6). ACE also affects the kinin–kallikrein system by inactivating bradykinin, a peptide known to induce vasodilation (6,7). Most of the recognized RAS components have already been detected in the human eye (8). In addition to circulatory RAS, there is a tissue-localized system, and local RAS may have a significant role in the formation of aqueous humor and also in its drainage (8). The encoding gene *ACE* in humans is located on 17q23, which is 21 kb in length, and includes 26 exons and 25 introns. Rigat et al. (9) identified a polymorphism in the ACE gene characterized by the insertion (I) or deletion (D) of a 287 base pair *alu* repeat sequence within intron 16. This polymorphism has consistently been associated with approximately 45% of the variance of serum ACE concentrations (9). Subject with the DD genotype have been found to have ACE levels twice as high as subjects carrying the II genotype (9). It has also been hypothesized that the up-regulation of the RAS system in hyperthyroidism and the increase in serum and tissue angiotensin II level may activate the synthesis of transforming growth factor-b (TGF-b), consequently resulting in hyaluronic acid accumulation in the orbital tissue (10). The purpose of this study was to test a possible association between *ACE* I/D polymorphism and TO in a Turkish population.

2. Materials and methods

2.1. Study population

A total of 105 patients with TO, diagnosed between December 2007 and December 2011, and 102 healthy persons were enrolled in this case-control study. The TO patients comprised 40 men and 65 women, aged 20–70 years (average age 42.17 ± 10.14 years). The healthy control subjects consisted of 50 men and 52 women, aged 31–57 years (average age 39.84 ± 10.40 years, P = 0.1042). The study was approved by the local Ethics Committee, and it was conducted in accordance with the Declaration of Helsinki. All study participants gave informed consent.

The patient group comprised individuals diagnosed with TO of at least 6 months' duration. Patients who had mild-to-moderate exophthalmoses, whose orbital soft tissue and extra-ocular muscles were affected, as documented by orbital magnetic resonance imaging, and whose illnesses were controlled medically, were included in this study. Ophthalmology grading was done according to the NOSPECS classification (11). No patient had a history of any corneal or optic nerve involvement, or prior orbital decompression surgery. Other than TO, no autoimmune or endocrine diseases were present in the patient group.

The control group comprised persons who had been referred to the outpatient service for a variety of conditions, including myopia, strabismus, and cataract. Persons with existing allergic/autoimmune diseases, e.g., asthma, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, were excluded from the study. Both the patients and control subjects were all Caucasian and Turkish descendants. None of the participants were related.

2.2. DNA isolation and genotyping

From all subjects, peripheral blood samples (5 mL) were collected by venipuncture into sterile siliconized Vacutainer tubes with 2 mg/mL disodium ethylenediaminetetraacetic acid. Immediately after collection, whole blood was stored at –20 °C until use. Genomic DNA was extracted from whole blood by standard proteinase K digestion and the salt–chloroform method (12), and stored at –20 °C. Analysis of the polymorphism of the ACE gene was performed as described previously (13). Both TO patients and control group subjects were analyzed for *ACE* I/D polymorphism using the polymerase chain reaction (PCR). The PCR products were visualized by electrophoresis in a 2% agarose gel with ethidium bromide and documented with a gel documentation system (Vilber-Lourmar, Eberhardzell, Germany) (Figure).

2.3. Statistical analysis

Results are expressed as the mean ± SD or percentage. Statistical analysis was performed using GraphPad Instat (version 3.05, GraphPad Software Inc., San Diego, CA, USA) statistical software. Polymorphisms were tested for deviation from Hardy–Weinberg equilibrium by comparing the observed and expected genotype frequencies using the chi-square test. For calculation of the significance of differences in genotype and allele frequencies, the chi-square test (with Yate's correction) or Fisher's exact test was used. The effects of genetic polymorphism on the risk of TO development were estimated by an odds ratio (OR) and its 95% confidence interval (CI). All statistical tests and P values were 2-sided, and $P < 0.05$ was considered to be statistically significant.

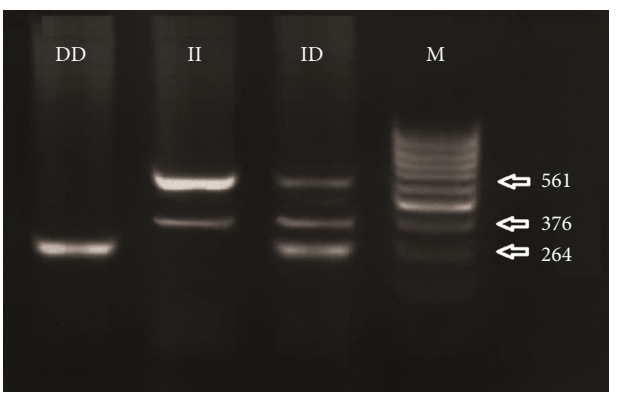

Figure. Agarose gel electrophoresis results. D allele reveals a band at 274 bp, and I allele reveals a 561 bp band. DNA fragment sizes, in bases, are indicated by numbers on right. M: size marker.

3. Results

The genotype distributions of *ACE* I/D polymorphism did not deviate from those predicted by Hardy–Weinberg equilibrium among cases (P = 0.961 for the TO group) and controls (P = 0.837). *ACE* genotype and allele distribution patterns for both groups are shown in the Table. Carriers of the DD, ID, and II genotypes were not found significantly more often among patients with TO than among control subjects. Similarly, no significant differences in the D or I allele frequencies of *ACE* I/D polymorphism were observed between patients and control subjects (Table). Subgroup analysis related to sex revealed no significant differences in the genotype and allele frequencies between patients and control subjects (Table).

4. Discussion

Numerous studies have previously investigated *ACE* I/D polymorphism as a potential risk factor for cardiovascular and endocrine diseases (14,15). Yet to the best of our knowledge, the present study is the first to evaluate the role of this polymorphism in patients with TO. No statistically significant association between TO and *ACE* I/D polymorphism was demonstrated. Our findings suggest that *ACE* I/D polymorphism was not involved in the TO pathogenesis for the Turkish population.

It is known that angiotensin II augments vascular permeability by stimulating the synthesis of vasoactive substances, e.g., vascular endothelial growth factor, leukotriene C_4, prostaglandin E_2, and prostaglandin I_2 (16,17). Angiotensin II also elevates the adhesion of macrophages and neutrophils to endothelial cells, and has a direct effect on chemotaxis, proliferation, and differentiation of immune system cells (17,18). Thus, angiotensin II is a potent and active pro-inflammatory mediator in the inflammatory cell infiltration of target cells/tissues in the continuum of the immune response (18). Signs and symptoms of TO also occur as a consequence of inflammation of the orbital connective/fatty tissue and soft-tissue enlargement in the orbit, leading to increased pressure within the bony cavity, inflammation with the production of cytokines and fibrosis of the extraocular muscles, and adipogenesis (19). ACE activity is known to be influenced by thyroid function at both the circulating and tissue levels. Hyperthyroid patients have shown an increase in serum ACE concentrations and activity that is positively correlated with thyroid hormone levels (20). The rate of preoperative high serum thyroid hormone levels was significant in patients with a benign thyroid pathology (21). Collectively, these data suggest that serum ACE levels increase during hyperthyroidism, and there is a strong correlation between ACE activity and thyroid hormone concentrations.

The volumes of both the extra-ocular muscles and retro-orbital connective and adipose tissues are increased,

Table. Genotype and allele frequencies of *ACE* I/D polymorphism in all patients with TO and controls. Frequencies according to sex are also shown.

Genotypes/ Alleles	Controls (n = 102) n (%)	Patients with OT (n = 105) n (%)	P value	OR (95% CI)
DD	42 (41.2)	36 (34.3)		
ID	44 (43.1)	49 (46.7)	0.485	1.299 (0.711–2.375)
II	16 (15.7)	20 (19.0)	0.464	1.458 (0.659–3.227)
D	128 (62.7)	121 (57.6)		
I	76 (37.3)	89 (42.4)	0.335	1.239 (0.835–1.838)
Female	(n = 52) n (%)	(n = 65) n (%)		
DD	24 (46.2)	20 (30.8)		
ID	19 (35.5)	31 (47.7)	0.162	1.958 (0.859–4.462)
II	9 (17.3)	14 (21.5)	0.305	1.867 (0.669–5.211)
D	67 (64.4)	71 (54.6)		
I	37 (35.6)	59 (45.4)	0.167	1.505 (0.886–2.556)
Male	(n = 50) n (%)	(n = 40) n (%)		
DD	18 (36.0)	16 (40.0)		
ID	25 (50.0)	18 (45.0)	0.822	0.810 (0.327–2.004)
II	7 (14.0)	6 (15.0)	1.000	0.964 (0.268–3.475)
D	61 (61.0)	50 (62.5)		
I	39 (39.0)	30 (37.5)	0.959	0.939 (0.512–1.719)

OR, odds ratio; CI, confidence interval

due to inflammation and the accumulation of hydrophilic glycosaminoglycans, principally hyaluronic acid, in these tissues (4,5). The accumulation of glycosaminoglycans induces a change in osmotic pressure, which in turn leads to a fluid accumulation and an elevation in pressure within the orbit and displace the eyeball forward in TO (4,5). Since components of the RAS system are localized in the human ocular tissues (8), and angiotensin II potently induces synthesis of transforming growth factor TGF-b (22), it is likely that increased angiotensin II levels may activate synthesis of TGF-b and then stimulate hyaluronic acid accumulation in the orbital tissue, as hypothesized by Sagheb et al. (10). There is also evidence that the inflammatory mediator TNF-a induces the formation of fibrotic foci by cultured retinal pigment epithelial cells through activation of TGF-b signalling (23), and serum TNF-a values are known to be increased in patients with hyperthyroidism (24).

It is known that thyroid-associated ophthalmopathy is more frequent in women (16 cases/100,000 people/year) than in men (3 cases/100,000 people/year) (25). We have also observed a high frequency of women patients in our study.

It is documented that *ACE* polymorphisms affect serum ACE levels and, thus, RAS activity and efficiency. The DD genotype and D allele of the ACE gene have been shown to be associated with higher enzymatic activity and expression (9). On the other hand, the I allele of the ACE gene has been shown to be associated with lower ACE activity and kinin degradation (26). However, we did not measure ACE enzyme activity or expression in this study.

It is known that ACE gene allele distributions vary greatly among different populations. The prevalence of the ACE gene polymorphism D allele is 39% in Japanese, 54% in European Caucasian, 56% in American Caucasian, and 60% in African American (27,28) populations. Our population consisted of Turkish Caucasians, and the genotype and allele frequencies found in this study were similar to the previously reported frequencies in Turkish populations (29,30). The D allele frequency has been reported to be 60.1% (29) or 64.0% (30) in healthy Turkish populations. We found a D allele frequency of 62.7% in the present study, which appears to be similar to the reported frequencies.

In conclusion, *ACE* I/D polymorphism is not a risk factor for TO in the Turkish population. The lack of association of *ACE* I/D genotypes and alleles between TO patients and control subjects leads us to exclude any possible relationship between *ACE* I/D polymorphism and TO. Studies in larger populations are needed to confirm these results in TO, and further studies are required to verify these findings in different ethnic groups.

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