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The evaluation of endothelin 1 (EDN1) and endothelin receptor type A (EDNRA) gene polymorphisms in Hashimoto's thyroiditis

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ABSTRACT

Purpose: Endothelin1 (EDN1) is well established marker of inflammation. The functions of EDN1 are mediated mainly by endothelin receptors type A (EDNRA). The etiopathogenesis of Hashimoto's thyroiditis (HT) remains still elusive although the role of chronic inflammation and subsequent endothelial dysfunction has been established. This study examined firstly the possible association of EDN1 (G5665Tand T-1370G) and EDNRA (C+70G and G-231A) single nucleotide polymorphisms (SNPs) with the occurrence of HT, and evaluates the relationship between genotypes and clinical/laboratory manifestation of HT.

Materials and methods: We analyzed genotype and allele distributions of above mentioned polymorphisms in 163 patients with HT and 181 healthy controls by real-time PCR combined with melting curve analysis.

Results: No significant associations between HT and variant alleles of EDN1 5665 and -1370, as well as EDNRA +70 and -231 SNPs were found. Haplotype analysis demonstrated that there was a strong ($D' = 0.76$, $r^2 = 0.487$) and weak ($D' = 0.403$, $r^2 = 0.086$) linkage disequilibrium (LD) between EDN1 -1370 and 5665, and between EDNRA -231 and +70 SNPs, respectively. However, haplotype frequencies in patients were similar to those in controls. In addition, it was observed that the EDNRA +70 G allele had protective effect against early (at age before 40 years) disease onset of HT (OR: 0.51, 95% CI = 0.32-0.79, $p = 0.003$).

Conclusion: Although no significant associations between susceptibility to HT with EDN1 5665 and -1370, as well as with EDNRA+70 and -231 SNPs were found, EDNRA +70 polymorphism was related with decreased risk for early onset HT.

1. Introduction

Endothelins (EDNs) are vasoactive peptides implicated in the inflammatory process, and having important cardiovascular, mitogenic and neuroregulatory functions [1–3]. There are three isopeptides identified — EDN1, 2, and 3. EDN1, a 21-amino acid peptide, is considered the most important vasoconstrictor and mitogen among the other isopeptides. The EDN1's effects are mediated predominantly by EDN1 specific EDNRA located on vascular smooth muscle cells [1,2]. Hashimoto thyroiditis (HT) is the most common organ-specific autoimmune disorder affecting approximately 18% of overall population [4]. It is characterized by diffuse lymphocytic infiltration of the thyroid gland, elevated levels of serum anti-thyroid antibodies, evidence of goitrous or atrophic gland, and frequent thyroid dysfunction in varying degrees [4]. The exact pathophysiologic mechanism of HT was not elucidated yet. Chronic inflammation, an imbalance between pro- and anti-inflammatory cytokines, and subsequent endothelial dysfunction are thought to play an important role in the etiopathogenesis [5,6]. Elevated plasma EDN1 [7] and increased expression of EDN1 and EDNRA in thyroid gland of patients with HT [8,9] have been described. EDN1 and EDNRA polymorphisms have been investigated in cardiovascular disease and hypertension [10–12], but there are also a few reports in some autoimmune diseases such as vitiligo [13], psoriasis [14], scleroderma [15], and primary biliary cirrhosis [16]. In our previous study, in firstly investigating for the association between EDN1 (G5665T and T-1370G), EDNRA (C+70G and G-231A) polymorphisms and susceptibility to Graves' disease (GD) — another autoimmune disease of thyroid gland [17], we found that there were no significant differences between allelic frequencies of the above-mentioned polymorphisms in GD and

Abbreviations: Anti-Tg, anti-thyroglobulin; Anti-TPO, anti-thyroid peroxidase; CI, confidence interval; GD, Graves' disease; EDN1, endothelin 1; EDNRA, endothelin receptor type A; HT, Hashimoto's thyroiditis; HWE, Hardy–Weinberg equilibrium; ICAM1, intercellular adhesion molecule 1; LD, linkage disequilibrium; NF-κB, nuclear factor-κB; OR, odds ratio; SNPs, single nucleotide polymorphisms; TSH, thyroid-stimulating hormone; VEGF, vascular endothelial growth factor; UTR, untranslated region.

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healthy controls. However, we have shown that EDN1 (G5665T and T-1370G) polymorphisms were related with alterations in autoantibody production, and EDNRA C+70G polymorphism was associated with GD ophthalmopathy.

Considering the increased EDN1 levels in HT patients, and the relationship between polymorphisms of EDN1 family genes with autoantibody production/GD ophthalmopathy, we aimed to investigate whether EDN1 (G5665T and T-1370G) and EDNRA (C+70G and G-231A) SNPs could predispose people to HT, and to evaluate the possible relationships between genotypes and clinical/laboratory findings of HT.

2. Materials and methods

One hundred and sixty three patients with the diagnosis of HT were included in the study. The diagnosis of HT was based on increased or normal TSH value, decreased or normal free T_3 or free T_4 values and increased titers of autoantibodies (anti-thyroid peroxidase antibody = anti-TPO, anti-thyroglobulin antibody = anti-Tg). The control group consisted of 181 individuals matched for age and sex. None of the controls had personal or family history of thyroid disease; they had normal thyroid functions and were negative for thyroid autoantibodies. Exclusion criteria were the existence of any comorbid cardiac, autoimmune, infectious, musculoskeletal or malignant disease and a recent history of operation or trauma. Characteristics of the HT patients and controls are shown in Table 1. The study was approved by the Institutional Review Board at Şişli Etfal Research and Training Hospital. Informed consent was obtained from each subject.

Blood samples were taken in the morning subsequent to an overnight (12 h) fast. Peripheral venous blood samples were collected in plain tubes for routine biochemical analysis. Serum triglyceride, cholesterol, HDL- and LDL-cholesterol measurements were performed on 1800 DPP Roche autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum TSH, free T_3, free T_4, anti-Tg and anti-TPO were measured on Modular EEE Electrode Elecsys Roche autoanalyzer (Roche Diagnostics, Mannheim, Germany).

Genomic DNA was isolated from peripheral blood leukocytes by using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). We examined the EDN1 G5665T (rs5370), EDN1 T-1370G (rs1800541), EDNRA C+70G (rs5335) and EDNRA G-231A (rs1801708) SNPs (Table 2). These SNPs were selected according to the following criteria: associated with susceptibility of other diseases [10–17], and adequate frequency in Caucasian populations to perform evaluation. For detection of the mentioned polymorphisms, Light SNiP assays were used. Light SNiP assays are based on simple probe melting curve analysis. They consist of pre-mixed primers and probes. They were developed and optimized according to NCBI "rs" numbers of mentioned single nucleotide polymorphisms (SNPs) by Tib MolBiol (Berlin, Germany). The detection of polymorphisms was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany).

All statistical analyses were performed with SPSS 15.0 for Windows (Chicago, IL, USA). Differences in genotype distributions and allele frequencies in the cases and the controls were compared for statistical significance using the chi-square (χ^2) test. The statistical significance for deviations from Hardy–Weinberg equilibrium (HWE) was determined using the Pearson χ^2-test. Odds ratios (ORs) were calculated and given with 95% confidence intervals (CIs). The wild-type genotype/allele served as a reference category. For numerical data, Mann–Whitney *U* and Kruskal–Wallis tests were used. Linkage disequilibrium (LD) and haplotype frequencies were estimated using the Haploview software and compared between cases and controls using a contingency χ^2-test [18]. In addition the NCSS 2000 statistical package (Kaysville, Utah, USA) was used to evaluate the power analysis. We had a 92% power to detect an effect size (W) of 0.20 using 2 degrees of freedom ($\alpha = 0.05$).

Table 1
Characteristics of controls and patients with Hashimoto's thyroiditis (HT; mean ± SD).

	Control (n = 181)	HT (n = 163)
Age (years)	39.5 ± 9.50	40.15 ± 9.10
HT onset		
<40 years, n (%)	–	97 (59.5)
>40 years, n (%)	–	66 (40.5)
Sex		
Male, n (%)	50 (27.6)	42 (25.8)
Female, n (%)	131 (72.4)	121 (74.2)
Family history, n (%)	–	73 (44.8)
Anti-TPO (IU/mL)	–	626.8 ± 382.4
Anti-Tg (IU/mL)	–	587.6 ± 454.5
Smoking, n (%)	81 (44.8)	85 (52.1)
TSH (mIU/L)	1.56 ± 0.9	4.6 ± 3.5*
FreeT_3 (pmol/L)	3.0 ± 0.4	3.1 ± 0.4
FreeT_4 (pmol/L)	11.2 ± 2.8	12.9 ± 3.5
Cholesterol (mg/dL)	179.54 ± 34.80	192.20 ± 34.51
Triglyceride (mg/dL)	104.16 ± 56.89	109.65 ± 56.28
HDL-C (mg/dL)	59.73 ± 11.16	59.21 ± 12.22
LDL-C (mg/dL)	108.00 ± 31.11	110.82 ± 30.86

Abbreviations: anti-TPO (anti-thyroid-peroxidase), anti-Tg (anti-thyroglobulin), TSH (thyroid stimulating hormone), HDL-C (high density lipoprotein-cholesterol), LDL-C (low density lipoprotein-cholesterol).
* Man–Whitney *U* test, $p < 0.05$.

Table 2
Single nucleotide polymorphisms (SNPs) in the endothelin 1 (EDN1) and endothelin receptor type A (EDNRA) genes.

Gene	Rs number	SNPs	Allele	Location
EDN1	5370	5665	G/T	6p24, exon 5
	1,800,541	−1370	T/G	6p24, promoter
EDNRA	5335	+70	C/G	4q31, exon 8, 3′-UTR
	1,801,708	−231	G/A	4q31, exon 1, 5′-UTR

3. Results

A total of 344 subjects were included in this case–control study. The mean age of HT patients was 40.15 ± 9.10 years (42 males, 121 females), and of controls was 39.5 ± 9.50 years (50 males, 131 females). Table 1 depicts the characteristics of the HT patients and controls. There were no significant differences among study and control groups in terms of mean age and sex distribution. No significant differences were observed between female and male patients with respect to clinical and hormonal parameters. Eighty five persons (52.1%) of the patients and 81 (49.7%) of the control subjects were smokers.

The genotypic and allelic distributions of the polymorphisms in the studied genes for patients and controls are shown in Table 3. All genotype distributions were in accordance with the HWE among the patients and controls. The allelic frequencies of EDN1 G5665T G (0.76) and T (0.24); EDN1 T-1370G T (0.80) and G (0.20); EDNRA G-231A G (0.64) and A (0.36); EDNRA C+70G C (0.54) and G (0.46) found in our control population were similar to those reported for the English [10,15], and German [11] populations, as well as in our previous study [17]. We did not find any associations between HT and variant alleles of EDN1 5665 (OR: 1.17, 95% CI = 0.83–1.65) and −1370 (OR: 1.27, 95% CI = 0.88–1.82), as well as EDNRA +70 (OR: 1.08, 95% CI = 0.80–1.46) and −231 (OR: 1.13, 95% CI = 0.83–1.53).

Lewontin's standardized disequilibrium coefficient (D′) was calculated as a measure for LD between the studied SNPs in the EDN1 and EDNRA genes (Fig. 1). EDN1 −1370 and 5665 were found to be in strong LD ($D' = 0.76$, $r^2 = 0.487$). In addition, a weak LD between EDNRA −231 and +70 was found ($D' = 0.403$, $r^2 = 0.086$). Haplotype frequencies are shown in Table 4. The most frequent haplotype among the patients and controls were EDN1 −1330/5665 TG (0.695 and 0.714, respectively), and EDNRA −231/+70 GG (0.363 and 0.361, respectively). There were not any significant differences in the haplotype frequencies between patients and controls.