

THE IMPACT OF THE D727E POLYMORPHISM HAS NO SIGNIFICANT ROLE IN MULTI NODULAR GOITER

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ABSTRACT

Interactions between individual genetic and environmental factors determine the onset of the multi nodular goiter (MNG). The thyroid-stimulating hormone receptor (*TSHR*) gene is a convincing candidate gene in the pathogenesis of certain thyroid diseases including MNG. We investigated the codon 727 polymorphism (p.Asp727Glu, p.D727E) of the human *TSHR* gene using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods in 31 Turkish patients with MNG and in 30 control subjects, aiming to evaluate the relationship between this polymorphism and MNG. After genomic DNA isolation, PCR amplification was performed using a pair of primers in exon 10 of the *TSHR* gene that contains the p.D727E polymorphism and digested by the *Nla*III (*Hin*III) restriction enzyme. We found the CC and CG genotype incidence for the patient group to be 0.71 and 0.29, respectively, and for the control group to be 0.8 and 0.2, respectively. No statistically significant difference was found between the genotype and allele distribution of both groups ($p = 0.417$ and $p = 0.449$, respectively). However, the polymorphism is significantly correlated with the low serum level of the TSH ($p = 0.047$). These results suggest that the p.D727E polymorphism of the *TSHR* gene

may not contribute to the pathogenesis of nontoxic MNG diseases.

Keywords: Multi nodular goiter (MNG); Codon 727 polymorphism; p.D727E; Thyroid-stimulating hormone receptor (*TSHR*) gene

INTRODUCTION

Goiter is the volumetric enlargement of the thyroid gland [1]. Benign nodular goiter is a heterogeneous thyroid disorder. It can be divided into solitary nodular and multi nodular thyroid disease. Multi nodular goiter (MNG) constitutes a mixed group of nodular entities. Structural and functional heterogeneity is the most characteristic feature of MNG [2,3]. The term toxic MNG comprises a spectrum of clinical entities, ranging from a single hyper functioning nodule within an enlarged thyroid gland that also contains non functioning nodules, to multiple hyper functioning areas scattered throughout the gland [4,5]. The thyroid-stimulating hormone (TSH, thyrotropin) controls thyroid function and growth. Its receptor (*TSHR*) activates the α subunit of the stimulatory G protein ($G_s\alpha$), leading to adenylate cyclase (AC) activation and cyclic AMP (cAMP) production [6,7]. The *TSHR* gene, located on chromosome 14q31, has been cloned and sequenced [8]. Polymorphisms have been detected in *TSHR* exon 10 [9,10]. The diallelic polymorphism in which a cytosine/ guanine base transition occurs at nucleotide position 2281 within codon 727 (c.2281 C>G), results in the substitution of glutamic acid for aspartic acid (p.Asp727Glu; p.D727E) in the intracellular portion of the receptor, has been associated with toxic or non toxic MNG, the expression of the p.D727E

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variant in eukaryotic cells resulting in an exaggerated cAMP response to thyrotropin stimulation. After TSH stimulation of the polymorphic variant according to the wild type *TSHR* gene increased cAMP accumulation. Thus, p.D727E could induce growth and function of thyrocytes [1]. We have investigated the frequency of this p.D727E polymorphism in Turkish patients with MNG and in control subjects, aiming to evaluate the relationship between this polymorphism and MNG.

MATERIALS AND METHODS

We studied 31 consecutive Turkish patients with MNG who underwent total thyroidectomy at the Department of General Surgery, Abant Izzet Baysal Medical School, Bolu, Turkey. The Abant Izzet Baysal University Ethics Committee had approved the study (#2009/100-78) and written informed consent was obtained from all subjects after full description of the objectives and procedures of the study.

Every patient underwent radioimmunology analysis of serum TSH, free thyroxine (FT4), free triiodothyronine (FT3) and thyroid autoantibodies (TPOAb, TGAb) before surgery and medical therapy for hyperthyroidism. According to the results of thyroid function tests, thyroid ultrasonography and a thyroid technetium 99 m (99mTc) scintigraphy, the patients were divided into toxic (subclinical hyperthyroidism and hyperthyroidism) MNG, and non toxic MNG. None of the patients had mental illnesses, systemic diseases (neurological or other endocrine), acute or chronic inflammatory or autoimmune diseases. Seventy-five percent of the patients have a positive family history of MNG.

Thirty healthy subjects attending the outpatient laboratory of the hospital with no history or evidence of MNG and no first-degree relatives with hyperthy-

roidism were recruited as control group. The patients and controls were unrelated Turkish subjects residing in the Bolu region, in northwest Turkey. The demographic characteristics and laboratory criterion of the patients and controls are shown in Table 1.

Venous blood (3 mL) was collected from subjects into EDTA-coated tubes, and genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). A pair of primers was designed to flank the region in exon 10 of the *TSHR* gene that contains the p.D727E polymorphism (rs1991517), as previously described [11]. To amplify the 196 bp DNA product containing the polymorphic site, primers 5'-CTC AGC AAG TTT GGC ATC TG-3'' (forward) and 5'-CTT CTG AGA TTT GGC CTT GC-3' (reverse) were used [<http://innere.uniklinikum-leipzig.de/tsh/frame.html>].

Polymerase chain reaction (PCR) was used to amplify genomic DNA in a 25 µL reaction volume containing 2.5 µL genomic DNA (20-50 ng), 1.5 µL MgCl₂ (25 mM), 1 µL dNTP (2.5 mM) 1 µL forward primer (20 ng/µL) and 1 µL reverse primer (20 ng/µL) and 0.1 µL Taq DNA Polymerase (5U/µL) (Vivantis Technologies, Selangor Malaysia), in the PCR buffer provided by the manufacturer (10mmol/L Tris-HCl, pH 8.0, and 100 mmol/L KCl). The PCR involved an initial denaturation at 95°C for 3 min., followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 4 min., using a thermocycler (Takara, Shiga, Japan). The PCR amplification product was 196 bp long. To detect the C>G substitution, we used the *Nla*III (*Hin*1III) (Fermentas, Vilnius, Lithuania) restriction enzyme. The G allele fragments were 129 and 67 bp long, while those for the C allele were 108, 67 and 21 bp long (Figure 1).

Table 1. The demographic characteristics of the patients and controls.

	Patients	Controls	p Value
Age (years)	53.03 ± 11.22 (36-77)	61.76 ± 6.80 (50-77)	0.001
Gender: males/ females	8/23	3/27	0.111
FT4 (ng/ dL)	1.41 ± 0.51 (0.95-3.19)	1.18 ± 0.15 (0.91-1.49)	0.059
FT3 (pg/ dL)	3.48 ± 1.54 (1.47-8.31)	2.86 ± 0.34 (2.39-3.63)	0.206
TSH (mIU/ L)	0.51 ± 0.68 (0.00-2.10)	1.56 ± 1.32 (0.17-6.10)	0.003
Family history of goiter:			
- positive	20 (65.0%)		
- negative	11 (35.0%)	30 (100.0%)	0.000

FT4: free thyroxine; FT3: free triiodothyronine; THS: thyroid-stimulating hormone (thyrotropin).

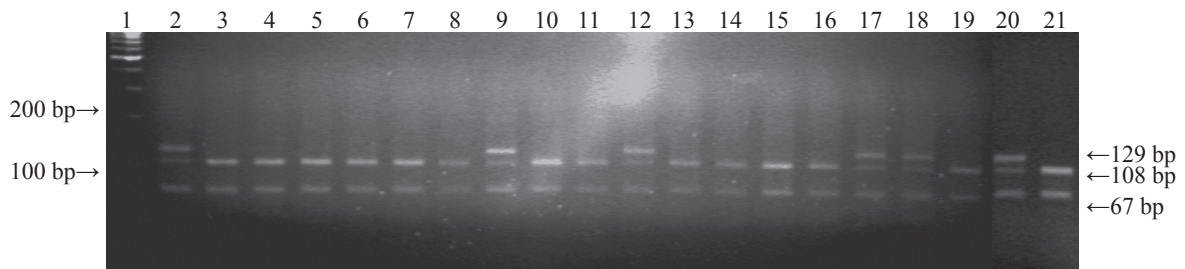


Figure 1. The PCR-based restriction analysis of the p.D727E polymorphisms of the *TSHR* gene shown on 2% agarose electrophoresis. The *Nla*III (*Hin*III) restriction enzyme cuts in the CATG/G region. In the presence of 129 and 67 bp, the fragments were shown to be GG (polymorphic homozygote) genotype; in the presence of 108, 67 and 21bp, the fragments were shown to be CC (wild type) genotype; in the presence of 129, 108, 67 and 21 bp, the fragments were shown to be CG (polymorphic heterozygote) genotype. Lane 1 was loaded with appropriate molecular markers; lanes 2, 9, 12, 17, 18, 20 are heterozygous CG subjects; lanes 3, 4, 5, 6, 7, 8, 10, 11, 13, 14, 15, 16, 19, 21 are homozygous CC subjects. A 21 bp fragment was not seen in either CC or CG genotypes.

Statistical analysis was done by using SPSS software package. Mann-Whitney U tests and Chi-square tests were used whenever appropriate and a *p* value of <0.05 was considered significant.

RESULTS

Clinical evaluation of the patients is shown in Table 2. Table 3 shows the distribution of the codon 727 genotypes of the *TSHR* gene (CG heterozygous; CC wild type) in patients and controls. In addition, the C and G allele frequencies of patient and control groups are given in Table 3.

Table 2. The clinical evaluation of the patients.

Parameters	Patients (%)
Thyroid disorder:	
- toxic MNG	48.39 (<i>n</i> = 15)
- non toxic MNG	51.61 (<i>n</i> = 16)
Thyroid function:	
- hyperthyroid	51.61 (<i>n</i> = 16)
- subclinical hyperthyroid	22.58 (<i>n</i> = 7)
- euthyroid	25.81 (<i>n</i> = 8)

MNG: multi nodular goiter.

Table 3. The distribution of the p.D727E genotypes and allelic frequencies of the *TSHR* gene in patients and controls.

	<i>Nla</i> III Genotype			Allele Frequency	
	C/C	G/G	C/G	C	G
Patients (<i>n</i> = 31)	70.97% (<i>n</i> = 22)	– (<i>n</i> = 0)	29.03% (<i>n</i> = 9)	53 (85.48%)	9 (14.52%)
Controls (<i>n</i> = 30)	80.00% (<i>n</i> = 24)	– (<i>n</i> = 0)	20.00% (<i>n</i> = 6)	54 (90.00%)	6 (10.00%)

CC: wild type genotype; GG: homozygous genotype; CG: heterozygous genotype.

We did not find a homozygous G allele (GG genotype) in our groups. There was no statistically significant difference between the genotype and allele distribution of the two groups (*p* = 0.417 and *p* = 0.449, respectively). Moreover, there was no significant difference in family history between the groups with respect to alleles (*p* = 0.959). However, the subtypes of toxic thyroid disease (toxic and subclinical MNG) were not related to significant differences in polymorphism frequencies compared with the non toxic MNG and control group, while the p.D727E polymorphism is significantly correlated with the low serum level of TSH (*p* = 0.047).

DISCUSSION

We screened the p.D727E polymorphism of the *TSHR* gene, a predisposition factor causing MNG. The statistical analysis of our data did not show a direct relationship between the p.D727E polymorphism and non toxic MNG in our groups. Clearly, the presence of the p.D727E polymorphism in the *TSHR* gene is not sufficient for the development of nodular goiter, because, according to our results, ap-

proximately 20.0% of normal individuals possess this polymorphism. In addition, despite a small number of patients, there was no correlation between the polymorphism and toxic MNG, however, frequency of polymorphism is associated with low TSH level.

Gabriel *et al.* [1] indicated that a p.D727E polymorphism of the human *TSHR* gene was significantly associated with toxic MNG. They argued that altered intracellular signaling of the variant receptor after stimulation by TSH might participate in the pathogenesis of toxic MNG. However, Gabriel *et al.* [1] reported that the presence of the heterozygous state for the p.D727E polymorphism of the *TSHR* gene was not solely responsible for the development of the disease, and that this variant existed in approximately 10.0% of normal individuals. This rate was higher than that in our control group. Also, Gozu *et al.* [12] reported similar geographic differences in the prevalence of the *TSHR* polymorphisms.

Thyrotropin receptor polymorphisms and thyroid hyper functioning after the first identification is still an unresolved question. Muhlberg *et al.* [13] studied the frequency of the polymorphism p.D727E in a large group of patients with non autoimmune hyper functioning thyroid disorders in a European Caucasian population. They demonstrate that there is no association with the gene polymorphism with toxic goiter. However, the presence of the *TSHR* polymorphism may predispose to hyper functioning, but in abnormal growth and nodular goiter pathogenesis, other genetic or environmental factors may be effective. However, in cases of less functional impairment, a contribution of variants of this gene in the etiology of non toxic goiter is possible.

In conclusion, taking into consideration the fact that the p.D727E *TSHR* variant is frequently detected in the general population, it would seem that it represents a simple polymorphism and probably is not involved in the development of thyroid diseases. However, positive family history of MNG in 65.0% of our patients suggests that there may be another related gene or genes responsible for the development of MNG in our population. This is the first study of the p.D727E polymorphism in Turkish patients with MNG. According to our data, the p.D727E variant is frequently detected in the general population; it would seem that it represents a plain polymorphism and is probably not involved in the development of structural thyroid disease. Further studies are nec-

essary to examine factors that orchestrate thyroid nodular transformations, and which genetic traits are associated with this process.

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