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An association study of genetic polymorphism of TSHR D727E with hypothyroidism: A study from central India

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Abstract
In present investigation, we conducted a case control-based genetic screening of single nucleotide polymorphisms (SNPs) loci in TSHR gene with the aim to explore the relationship between the candidate SNPs and the susceptibility to hypothyroidism (HPO) in central India. A genetic polymorphism study of TSHR was undertaken using a polymerase chain reaction-restriction fragment length polymorphism method the patterns of genotype and allele distribution in both groups suggested a non-significant association between TSHR D727E gene polymorphism hypothyroidism in central India population. Body mass index was found to be significantly higher in female and male HPO patient group than in female and male healthy controls ($P = 0.0001$).

Keywords: hypothyroidism, BMI, PCR-RFLP, thyroid stimulating hormone receptor (TSHR)

Introduction

Hypothyroidism is described as the high level of serum TSH and normal or low level of thyroid hormones (T3 and T4), one of the most frequent thyroid disorders worldwide and is an important and continuing health problem globally. Thyroid disorder has been reported in over 110 countries of the world with 1.6 people at risk (Khan *et al*., 2002) [21]. According to various studies, 200 million individuals worldwide and 42 million individuals in India have a thyroid disorder (Unnikrishnan *et al*., 2011 and Ahmed *et al*., 2016) [35, 2]. Data from Whickham survey showed an annual incidence of hypothyroidism is 4.1 per 1000 in women and 0.6 per 1000 in men (Chekar *et al*., 2012) [8]. Iodine deficiency and in area where iodine intake is adequate autoimmunity, radiation and drugs are the commonest causes of thyroid disorder worldwide, while factor like age, sex, obesity, pregnancy, ethnicity and geographical location play an essential role in the prevalence (Chandey M *et al*. 2016) [9] and it have tendency to run in families. Hypothyroidism is associated with a collection of signs, symptoms and long-term complications such as skin manifestations, obesity, hyperlipidemia, bradycardia, fatigue and depression (Sayer *et al*., 2014) [34].

Genetic and Environmental factor (lifestyle factor) play an important role in defining thyroid function throughout life, both in health as well as in disease states. The genome scanning and single nucleotide polymorphisms (SNP) studies have made great progress in the identification of susceptibility genes (Eric *et al*., 2007; Eriksson *et al*., 2012) [15, 16]. One of the candidate genetic risk factors for development of thyroid disease is TSHR, encoded by the TSHR gene is located on chromosome 14q31 and contained 10 exons and 9 intron, is a G-protein-coupled receptor with a seven-transmembrane domain (TMD) activating the classical G-protein-coupled effectors, adenylate cyclase (AC) and phospholipase C (PLC) and a large extracellular domain (ECD) responsible for high-affinity hormone binding. TSHR is 764 amino acid sequence with large glycosylated ECD of 395 residues is encoded by the first 9 exons and part of exon 10, whereas remaining 349 residues, encoded by the 10th and large exon constitute the seven transmembrane domain (TMD) and intracytoplasmic tail (Peeter *et al*., 2006; Sayer *et al*., 2014; Cassio *et al*., 2013 and Gozu *et al*.,2016) [27, 34, 7, 20]. Many studies have investigated the role of mutations in gene encoding. TSHR in the development of different thyroid diseases (Louwerens *et al*., 2012) [25].

Three germline TSHR polymorphisms have been identified: Asp36His, Pro52Thr, and Asp727Glu (Peeter *et al*., 2006) [27]. The TSHR-Asp727Glu polymorphism is one of the diallelic polymorphisms of TSHR gene a cytosine/guanine base transition at nucleotide position 2281 with codon 727 resulting in the substitution of glutamic acid for aspartic acid (D727E) within the intracellular portion of the receptor (Chou *et al*., 2002). A role for TSHR in some forms of hypothyroidism has been established. Germline loss-of-function mutations of the TSHR are associated with TSH resistance and hypothyroidism (Abramowicz *et al*.,1997; Cordella *et al*., 2007 and Cassio *et al*.,2013) [1, 12, 7] in which the binding or signaling functions of the receptor are diminished will lead to a state in which the thyroid gland is underdeveloped and unable to produce sufficient thyroid hormone to maintain a euthyroid state. Germline loss of function mutation of TSHR gene is associated with slightly elevated level of TSH and other studies have investigated the role mutation in TSHR gene in development or their association with different thyroid disorder and other clinical endpoints such as osteoporosis, either via its influence on thyroid hormone levels, or via direct effects of TSH on bone. (Liu *et al*., 2012) [24]. Many previous reported that the effect of the TSHR Asp727Glu polymorphism on various clinical outcomes, including bone metabolism, glucose metabolism, and preeclampsia. Some studies have reported the prevalence of fatigues with hypothyroidism and significant association with TSHR D727E polymorphism.

Thyroid stimulating hormone receptor (TSHR) is thought to

play a critical role in the pathogenesis of certain thyroid diseases, including Graves' disease (GD) (Brand *et al*., 2009) [6], multinodular thyroid goiter (MTG), TMNG (Gabriel *et al*. 1999) [19], Hashimoto's thyroiditis and autoimmune thyroid disease(Lin *et al.*, 2012) [23] and Congenital hypothyroidism(CH)(Musa *et al*., 2008, Ma *et al.*, 2010) [32, 30]. In order to understand whether single nucleotide polymorphisms in the TSHR gene contribute to hypothyroidism, we have conducted a case–control study in which, we examined TSHR D727E gene single-nucleotide polymorphisms among patients with hypothyroidism.

Materials and Methods

Subjects

The study was design as a case-control study. Case group were consisted of 174 HPO patients aged between 25 and 61 years (mean of 41.29 years), including 24 men and 150 women and control group consisted healthy individuals.

Sample collection

About 5 ml venous blood samples were obtained from all the cases and controls and distributed into anticoagulant free plain tube (3 mL) and evacuated EDTA tube (2 mL). The blood sample in the plain tube was centrifuged after 30 minutes of sampling and serum was isolated and stored at -20°C. Blood sample was collected in EDTA vial for genetic analysis and store at -20°C till analysis.

Measurements of body mass index (BMI) and blood pressure

BMI was calculated as (weight in Kg) / (height in meters)^2 and Waist circumference was measured in standing position midway between iliac crest and lower costal margin. Blood pressure was measured twice in the right arm in sitting position after resting at least 5 minute using a standard sphygmomanometer and average of the two reading was used.

Thyroid Profile Estimation

Thyroid profile estimation related to hypothyroidism were estimated for both cases and controls subjects**.** Measurement of Serum levels of thyroid hormones (freeT3 and freeT4) and TSH were measured in each participant using ELISA (Enzyme Linked Immuno sorbent Assay) technique by (Aspen Laboratories Pvt. Ltd.).

DNA Extraction

The venous blood samples were collected in the evacuated EDTA tubes. DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988) [31].

Molecular Analysis

Polymorphism screening via PCR-RFLP

PCR-based restriction enzyme analysis was performed in the isolated DNA (Liu *et al.*, 2012) [24]. The presence of DNA was confirmed by 0.7% Agarose gel electrophoresis and the amount of DNA was quantified using Nano-200 Micro-Spectrophotometer (Hangzhou Allsheng Instruments Co. Ltd.).

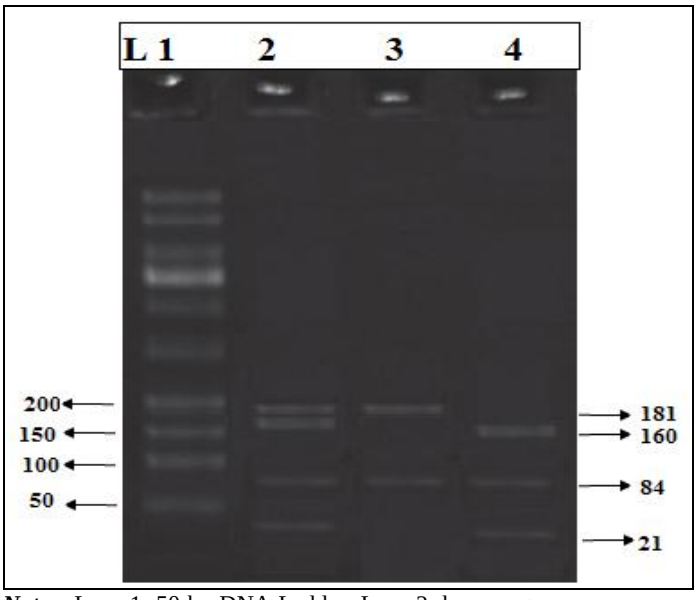
Polymerase Chain Reaction Analysis (PCR)

The DNA sequence (265 bp) of TSHR D727E gene was amplified by PCR. Amplification of TSHR sequence were performing in 25 µl of reaction mixture containing the following ingredients: Genomic DNA 40 ng, PCR buffer (Biotool,B and M Labs SA Spain), dNTP (200 µM)(HIMEDIA), $MgCl_2$(1.5mM)(Biotool, B and M Labs SA Spain), forward(5'-CCATTCCTCTATGCTATTTTCAC-3') and reverse (5'-CCGTTTGCATATACTCTTCTG-3') primers (Eurofins Genomic India Pvt Ltd), 4 pmol each for the TSHR gene D727E Single Nucleotide Polymorphism (SNP) (D727E), and 0.5 Units of Taq DNA polymerase (3B BlackBio Biotech India Ltd).

Amplification for the TSHR D727E SNP was performed with an initial denaturation of 95 ºC for 2 min in BIO-RAD S1000 thermal cycler (Bio-Rad Laboratories Inc.). The PCR amplification conditions were as follows: 35 cycles consisting of 60 s denaturation at 95°C, 1 minute annealing at 50°C, 60 s extension at 72°C, followed by final extension at 72°C, for 10 min. The PCR amplified product (265) was confirmed by 2% agarose gel electrophoresis under UV-transilluminator.

Restriction Enzyme Analysis

The amplification product of 265bp was digested by the specific restriction enzyme, 10 U of NIaIII for 16 at 37°C. PCR products and restriction fragments were separated by electrophoresis in agarose gel of appropriate concentration and stained with ethidium bromide. In presence of allele D, recognize 2 cutting site and cleave 265 bp PCR product into 160, 84 and 21 bps but in mutant status it produce two 181 and 84 bp fragments while in DE heterozygote genotype gives 181, 160, 84 and 21 bp fragments. Samples were analyzed by electrophoresis using 2.5% agarose gels to determine the genotype pattern of the gene. The results were documented by digital camera and further recorded by gel documentation system (Figure 1).



Notes: Lane 1: 50 bp DNA Ladder; Lane 2: homozygous mutant genotype EE; Lane 3: heterozygous genotype DE; Lane 4: wild-type genotype DD

Fig 1: Representative gel electrophoresis picture of TSHR D727E polymorphism.

Statistical Analysis

Mean and Standard deviation was calculated for anthropometric parameters and hormone profile for both the healthy individuals and HPO patients. Student t test was performed to find difference in the anthropometric parameters and hormone profile between the healthy individuals and HPO patients. Statistical analysis was performed using Student's *t*-test and the *P* values obtained suggested the level of significant change. Genotype, allele frequency, and carriage rate were analyzed using the chi-square test and Fisher's exact test, with *P* values, odds ratios (ORs), and confidence intervals recorded. All analyses were undertaken using Prism software (v 3.0; GraphPad, San Diego, CA, USA).

Result

The descriptive data and comparison of anthropometric parameters of HPO patients versus controls are presented in Table 1. As expected, both female and male HPO patients weighed markedly high than healthy controls ($P = 0.0001$ and $P = 0.0001$, respectively) and women in this group had higher BMI than women in the control group ($P = 0.0001$). Moreover, HPO female subjects had a greater waist circumference than controls ($P= 0.0001$) and. There were no other significant anthropometric differences between the groups **(Table 1**)

Biochemical and clinical findings

The descriptive data and comparison of biochemical parameters for patients versus controls are presented in Table 2. As expected, patients of hypothyroidism had markedly higher levels of serum TSH ($P = 0.0001$) and low FreeT3 ($P = 0.0001$) and Free T ($P = 0.0001$) compared with those of controls. Nominal differences were also observed for systolic blood pressure ($P = 0.2660$) and diastolic blood pressure ($P = 0.3711$,). All clinical test results are tabulated in Table 2.

Table 1: Comparison of anthropometric parameter of HPO patients and controls

Characteristics	Cases	Controls	P-value
n(Men/Women)	174(24/150)	200(100/100)	
Age(years)	$41.29^{\pm 9.127}$	$35^{\pm 9.172}$	P< 0.0001
Height(m)	1.613±0.5548	1.664± 0.08321	0.200
Weight (Kg)			
Women	81.81± 9.672	$67.49^{\pm 9.871}$	P< 0.0001
Men	84.12 ±8.341	$73.28^{\pm 8.004}$	P< 0.0001
BMI (kg/m^2)			
Women	$31.89^{\pm 0.04379}$	$26.38^{\pm 3.956}$	P< 0.0001
Men	$29.65^{\pm 2.972}$	$24.58^{\pm 2.369}$	P< 0.0001
Waist circumference (cm)			
Women	$102.3^{\pm 9.926}$	$91.16^{\pm 11.93}$	P< 0.0001
Men	103.9 ±12.24	$86.98^{\pm 7.187}$	P< 0.0001

Table 2: Comparison of Biochemical and clinical finding of HPO patients and controls

Characteristics	Case	Control	P – value
TSH (ml/dl) Mean ± SD	$24.16^{\pm 20.37}$	$2.69^{\pm 0.983}$	P<0.0001***
T3 (pmol/l) Mean ± SD	$3.658^{\pm 1.093}$	$4.453^{\pm 0.757}$	P<0.0001***
T4 (pmol/ll) Mean ± SD	$12.12^{\pm 3.522}$	$14.11^{\pm 3.060}$	P<0.0001***
Systolic BP(mmHg)	$123.4^{\pm 7.975}$	$122.5^{\pm 76.31}$	0.2660
Diastolic BP(mmHg)	$77.17^{\pm 9.664}$	$76.31^{\pm 8.902}$	0.3711

*denotes the level of significant change between case and control

Table 3: Prevalence of the genotypes and alleles in D727E position in both patient and control group.

Tshr Genotype		Case N= 174	Control N=200	Chi- Square Value χ^2 (P Value)
		N %	N %	
DD		153 87.93	170 85	0.6818 (0.7111)
DE		19 10.92	27 13.5	
EE		2 1.15	3 1.5	
Alleles	D	325 93.39	367 91.75	0.7234 (0.3950)
	E	23 6.61	33 8.25	
Carriagerate	D	172 89.12	197 86.78	0.5331(0.4653)
	E	21 10.88	30 13.21	

Notes: *$P<$ 0.05; **$P<$ 0.01; ***$P<$0.001. "%" indicates genotype allele frequency and carriage rate expressed as a percentage.

Detection of genetic polymorphism in TSHR

The genotype distribution of was strongly under the Hardy–Weinberg equilibrium ($\chi 2$ = 3.34 and 0.26 for cases and controls, respectively). No significant level of change has been seen in distribution of TSHR D727E genotypes in Healthy Control (HC) group as compared to HPO(Hypothyroidism) group although HPO group showed little increase in common DD genotype as compared to control (87.93% *vs.* 85% respectively). Similarly, EE genotype was present at lesser frequency in the case group 1.15 % and also in control group 1.5%. The overall genotype was statistically nonsignificant (χ^2 =0.6818, P=0.7111). DD genotype frequency was higher in case group and the odds ratio of DD genotype and DE genotype was 1.286 and 0.7854 respectively indicate little or no effect and association of this polymorphism with the hypothyroidism susceptibility.

Major allele D was found at slightly higher frequency in HPO group (93.39 %) as compared to HC group (91.75%) whereas allele E was present in slightly higher frequency in the control group (8.25 in control and 6.61 % in case) but the difference was nominal and not statistically significant (χ^2 =0.7234, P=0.3950). An odds ratio of 1.271 of major allele D shows moderate effect of major allele in hypothyroidism susceptibility. Carriage rate of allele E was slightly higher in control group as compared to case (13.21% Vs 10.88%) whereas carriage rate of allele D was slightly higher in case group as compared to control (89.12% vs 86.78%) and no significant level of change has been seen. The Odds ratio of allele D carriage is 1.247 which did not suggest any association of D allele carriage with disease susceptibility. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests TSHR D727E is not significantly associated with hypothyroidism in our population.

Discussion

Hypothyroidism is a multifactorial common thyroid disorder worldwide as well as in India. It is strongly associated with genetics as well as environmental factors(lifestyle factor).The present study, performed in a population of subjects all effected by increase serum TSH level with low or normal serum free T3 and free T4 level and demonstrated that individual affected by overweight and obesity are a higher prevalence with hypothyroidism.

BMI is used as a screening tool to indicate whether a person is underweight, overweight, obese or a healthy weight for their height. Obesity is one of most important global health problem at present and by common practice, measured in the Western world by a Body Mass Index (BMI) of 30 kg/m^2 or greater (Rotondi *et al.,* 2009) [29]. Danforth *et al.*(2002) [14] reported that even mild thyroid dysfunction in form of subclinical hypothyroidism linked to significantly change in body weight and connected with overweight and obesity. In our investigation, we found that BMI was significantly higher in patients of hypothyroidism in both female and male. The BMI averages of case were 31.89 as compared with 26.38 in control (*P* = 0.0388). Waist circumference was significantly higher in both males and females in the case group. A sedentary lifestyle is a key to the rise in the prevalence of both obesity and thyroid disease (Farasat *et al.,* 2011) [18].

Hypothyroidism is associated with weight gain with slight variation in thyroid function contribute to the development of regional obesity and tendency to gain weight but all studies have not been confirmed it (Biondi *et al.,* 2010) [5]. An elevated serum TSH level with normal peripheral thyroid hormone concentration suggesting subclinical hypothyroidism has been consistently found in obese subjects (Elzbieta Bandurska-Stankiewicz, 2013) [17]. De Pergola *et al.,* (2014) found in their study that overweight and obesity increase the risk of thyroiditis and subclinical hypothyroidism. Previous studies shows that the degree of obesity as measured by BMI was significantly positive associated with higher level of TSH level (Knudsen *et al*., 2005 and Bastemir *et al*., 2007) [22, 4]. Our data also suggest that higher BMI and obesity was strongly associated with hypothyroidism.

The physiological role of the TSH receptor (TSHR) as a major regulator of thyroid function by controlling the size and number of thyroid cells (thyrocytes) and exerts its effects through binding of TSH. Although primarily expressed on thyroid follicular cells, evidence suggests TSHR expression in the anterior pituitary, adipose tissue, retro-orbital tissue, osteoblasts, the immune system, and cardiac muscle. TSHR D727E common genetic variation which is associated with certain thyroid disease.

Our findings show that the genotype pattern and allele frequency of TSHR D727E was not significantly different between case and healthy controls. The homozygous DD genotype was more common in both group case (153/174) and control (170/200) compared to heterozygous DE and homozygous EE. The DE genotype was found 19 in case and 27 in control. Fisher's exact test was used to compare the genotype distribution and individual allele frequencies between case and control. There was no significant difference in either the genotype distribution or allelic frequencies between case and control (P= 0.7111 and P=0.3950 respectively). The frequency of the homozygous mutant 727E allele was very low in cases as well as in controls. Overall, the allele D was found at a slightly higher frequency in the disease group than in the healthy control group, whereas allele E was present at a slightly higher frequency in the control group (10.88% in patients and 13.21% in controls) but the difference was nominal. An OR of 0.7870 for the rare allele E and OR of 1.271 for the allele D shows little or no effect of the TSHR D727E polymorphism in hypothyroidism susceptibility. The carriage rate of allele D was slightly higher in the case group than in the healthy control group (89.12% Vs 86.78%).

Previous studies have reported that the D allele of the D727E polymorphism of the *TSHR* gene was significantly associated with Congenital Hypothyroidism(CH) in the Indian but not in the Malay and Chinese population in the cohort of Malaysian patient with CH (Musa *et al*., 2008) [32]. In this study we also found slightly higher frequency of major allele D was present in case group but its effect or association with hypothyroidism was not significantly established. Alves *et al.,* (2010) [3] found high frequency of D727E polymorphism in TSHR gene in Brazilian patients with Congenital hypothyroidism and Ma *et al*., (2010) [30] found that heterozygous genotype has normal thyroid hormone level and slightly elevated level of circulating TSH (5.96-6.92 mU/L) in 6 family members of Chinese children with CH.

In contrast, Chistiakov *et al*., (2002) [10] have reported that the E allele and the heterozygous DE genotype were significantly

more frequent ($p<0.0001$) in patients with GD than in control subjects of the Russian population and suggesting that the E allele and the C/G genotype are related to higher risk of GD. In addition, the heterozygous DE genotype is associated with TMNG (P=0.019) but not Grave's disease, in the US population (Gabriel *et al*., 1999) [19] suggesting that the presence of the DE genotype is an important predisposing genetic factor in the pathogenesis of TMNG. Gabriel *et al.,* (1999) [19] failed to detect a significant difference in codon727 polymorphism between patients with toxic multinodular goiter and the healthy control group while Gozu *et al. (2016)* [20] reported that high frequency of a germline polymorphism of codon 727 of TSHR in patients with toxic multinodular goiter in Turkish patients, suggesting that it may be a predisposing factor in toxic multinodular goiter. According to study of Muhlberg *et al*.,(2000) [26] subtypes of toxic non autoimmune thyroid disease (toxic adenoma, 13.2%; multinodular goiter, 9.6%; disseminated autonomy, 21.4%) were not related to significant differences in codon 727 polymorphism frequencies compared with the healthy control group (P = 0.67, P = 0.40, and P = 0.70, respectively) in a European Caucasian population.

Previous reports have assessed the effect of the TSHR-Asp727Glu polymorphism on various clinical outcomes, including bone metabolism, glucose metabolism and preeclampsia. Procopciuc *et al*.(2011) [33] evaluated that higher TSH levels and/or the TSHR-Asp727Glu polymorphism represent risk factors for preeclampsia and could be correlated with the severity of preeclampsia in Romanian population while Peeters *et al.* (2007) [28] examined TSHR-Asp727Glu, polymorphism and concluded that Asp727Glu was associated with IR in healthy elderly males. Taking this finding together with the increased Insulin Resistant(IR) risk in Subclinical Hypothyroidism, it is possible that individuals with TSHR-Asp727Glu can express TSHRs with a higher affinity to TSH, which can also result in increased TSHR signaling mimicking the SH situation, and thus are predisposed to IR. Liu *et al* (2012) [24] revealed that TSHR gene D727E polymorphism is associated with osteoporosis in China.

In conclusion, taking into consideration the fact that the 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 in our population. This is the first study of the D727E polymorphism in central Indian patients with hypothyroidism. According to our data, the 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 hypothyroidism in population Central India.

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