POLYMORPHISMS IN TSHR GENE AND THE RISK AND PROGNOSIS OF AUTOIMMUNE THYROID DISEASE IN TUNISIAN POPULATION

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Abstract

Background. Autoimmune thyroid diseases (AITD) including Graves' disease (GD) and Hashimoto's thyroiditis (HT) are complex genetic diseases. TSHR is considered as candidate gene in GD. This finding prompted us to investigate the association of TSHR gene polymorphism with the risk and the prognosis of AITD in Tunisia.

Methods. A total of 84 healthy controls and 91 patients with AITD (69 HT and 22 GD) were genotyped for TSHR rs74067403A>G polymorphism and 134 healthy controls and 149 patients with AITD (98 HT and 51 GD) were genotyped for TSHR rs1054708 T>C polymorphism.

Results. For rs1054708, we found an association between HT, AITD and the heterozygous genotype TC, the mutated genotype CC and the genotypes presented the mutated allele C (TC+CC) and with mutated allele C. The heterozygous genotype TC and the genotypes that presented the mutated allele C of rs1054708 are associated with male patients with AITD even the heterozygous genotype TC is associated with age of onset of disease.

Conclusions. These preliminary results suggest that TSHR rs1054708 polymorphism may be a protective factor against HT and AITD. This polymorphism can affect the etiology of AITD between men and women and also by age.

Key words: AITD, Anti-thyroglobulin antibody, Anti-thyroid peroxidase antibody, TSHR.

INTRODUCTION

Autoimmune thyroid diseases (AITD) are common autoimmune diseases, affecting up to 5% of the general population (1). During AITD the immune system attacks the thyroid gland. During Graves' disease hyperthyroidism is due to thyroid-specific auto-antibodies (thyroid-stimulating hormone receptor TSHR) production, while during Hashimoto thyroiditis, hypothyroidism is due to lesions of the thyroid gland

by anti-thyroglobulin (Tg) and anti-thyroid peroxidase (TPO) antibodies (2, 3). Several studies have shown that immune regulatory genes and thyroid-specific genes contribute to AITD susceptibility (4, 5).

The thyrotropin receptor gene is located on chromosome 14q31 and covers a 191.1 Kb region of the human genome (6, 7). The TSHR protein is a major auto-antigen in Graves' disease (8). The TSHR gene contains several polymorphisms which have been described. The rs74067403 (c.179654 A>G) and the rs1054708 (c.193335 T>C) are two dimorphisms in this gene.

For this we have chosen to study the association of two dimorphisms of TSHR gene with the prognosis of AITD in the Tunisian population.

SUBJECTS AND METHODS

Thyroid-stimulating hormone receptor rs74067403 polymorphism

Patients and controls

Eighty-four healthy controls with no family history of any autoimmune diseases and 91 patients with AITD (69 with HT and 22 with GD) are collected from the middle coast of Tunisia.

The AITD patients (85 women and 6 men) have an average age of 46 ± 12 years. The average age of patients with HT (65 women and 4 men) and with GD (20 women and 2 men) is 46 ± 12 years. The average age of control individuals (3 men and 81 women) is 46 ± 12 years.

Graves' disease and Hashimoto's thyroiditis were diagnosed by standard criteria set by the American Association of Clinical Endocrinologists and the American Thyroid Association (AACE/ATA) (clinical examination, thyroid function tests, thyroglobulin and thyroid peroxidase antibody titres and scintigraphy) (9, 10). The diagnosis of Graves' disease is based on a

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suppressed serum thyroid stimulating hormone (TSH) in addition to clinical results. HT is diagnosed alongside clinical results, by a high serum TSH level (> 5 mIU/L), a low serum FT4 level (< 8.6 pmol/L) and serum antibodies positive against TPO and Tg. All patients and controls have given written informed consent.

Genotyping of thyroid-stimulating hormone receptor rs74067403 polymorphism

The salting out technique is used to the genomic DNA extraction (11). For the rs74067403 polymorphism was analyzed using specific primers 5'-CAGTTGAATGCCATGTCTGG-3' and 5'-GCATGGTGGAAGTGAAAAC-3'. An Applied Biosystems™ 2720 thermal cycler is used to perform PCR. We have used the following program: 5 minutes of initial denaturation at 94°C followed by 35 cycles each of which consists of one minute of denaturation at 94°C, one minute of primer annealing at 55°C and one minute of elongation at 72°C. The whole is followed by 10 minutes of final elongation at 72°C. The mixture contained 50 ng of genomic DNA, 0.6 µmol of each primer, a 10X PCR buffer, 2 mM MgCl₂, 200 mM from each dNTP and 1 unit of Ampli Taq DNA polymerase. The PCR product is then visualized on 2% agarose gel stained with ethidium bromide.

The enzymatic digestion was carried out using the enzyme PstI (5 units/ µL) incubated for 14-16 hours at 37°C. The digestion product is revealed on a 3% agarose gel stained with ethidium bromide.

Thyroid-stimulating hormone receptor rs1054708 polymorphism

Patients and controls

One hundred thirty-four healthy controls with no family history of any autoimmune diseases and 149 patients with AITD (98 with HT and 51 with GD) are collected from the middle coast of Tunisia.

The AITD patients (134 women and 15 men) have an average age of 46 ± 12 years. The average age of patients with HT (91 women and 7 men) and with GD (43 women and 8 men) is 46 ± 12 years. The average age of control individuals (4 men and 130 women) is 46 ± 12 years.

All patients and controls have written informed consent.

Genotyping of thyroid-stimulating hormone receptor rs1054708 polymorphism

The salting out technique is used to the genomic DNA extraction (11). The rs1054708

polymorphism was analyzed using specific primers 5'-TGCTTCCTTTGGTGGGAATA-3' and 5'-ACCAGCAAGATTTTGGAGTTG-3'. PCR was performed using Applied Biosystems™ 2720 Thermal Cycler and according to the following program: an initial denaturation at 94°C for 5 minutes followed by 35 cycle search consisting of denaturation for 1 minute at 95°C, a primer annealing at 52°C for 1 minute and elongation for 1 minute at 72 °C. After 35 cycles, the PCR is terminated by a final elongation of 10 minutes at 72°C. The PCR cocktail contained 50 ng of genomic DNA, 0.6 µmol of each primer, 10X PCR buffer, 2 mM MgCl₂, 200 mM of each dATP, dGTP, dTTP, dCTP, and 1 unit of AmpliTaq DNA polymerase. The verification of the amplification and the specificity of the reaction are carried out by electrophoresis on an agarose 2% gel stained with ethidium bromide (12).

The enzymatic digestion was carried out as above.

Thyroid function and autoantibodies

The sera were obtained from each GD and HT patients at the onset of the disease. The measurements of serum thyroid stimulating hormone (TSH), Tetraiodothyronine (T4), anti-thyroglobuline (anti-Tg) and anti-thyroid peroxidase (anti-TPO) were performed by the clinical chemistry laboratory of the University hospital Fattouma Bourguiba, using commercial reagent kits following the manufacturer's instruction (12).

The normal range of serum TSH is 0.15-5mIU/L and of FT4 is 8.6-25 pmol/L. For anti-Tg and anti-TPO a reciprocal titer of $> 1:100$ was considered positive (12).

Statistical analysis

Allele and genotype distribution between groups were evaluated using Chi-square test or Fischer exact test. The difference in frequencies between the case and control groups was analyzed for statistical significance at 95% confidence interval using χ^2 test and Yates' correction. The allele's frequencies of TSHR gene were in Hardy-Weinberg equilibrium. Odds ratios (OR) at 95% confidence intervals were calculated. Each clinical feature is compared with TSHR genotype counts in the AITD, HT and GD patients using the following segregations: age of patients (< 40 years old vs. ≥ 40 years old), sex (women vs. men), thyroglobulin antibody and thyroid peroxidase antibody (positive vs. negative). Mean and standard deviation were calculated for FT4 and TSH hormone profile for GD and HT patients. ANOVA test was performed to find out whether there was a significant difference in

Figure 1 a. Polymorphism chain reaction amplification bands for TSHR polymorphism rs74067403 (c.179654 A>G). Lane N shows DNA size marker and lane B shows negative control (no DNA).

Figure 1 b. Electrophoresis of enzymatic digestion for TSHR polymorphism rs74067403 (c.179654 A>G). Lane M shows DNA size marker.

Table 1. Distribution of rs74067403 of TSHR genotypes and alleles in patients with AITD (n=91), HT (n=69), GD (n=22) and healthy control subjects (n=84)

	Control n (%)	AITD n (%) p-value	HT n (%) p-value	GD n (%) p-value
Genotype				
AA	27 (32.14)	34 (37.36)	26 (37.68)	8(36.36)
AG+GG ^a	57 (67.86)	57 (62.64) ns	43 (62.32) ns	14 (63.63) ns
Allele				
A	111 (66.07)	125 (68.68)	95 (68.84)	30 (68.18)
G	57 (33.93)	57 (31.32) ns	43(31.16) ns	14 (31.82) ns

AITD = autoimmune thyroid disease, GD = Graves' disease, HT = Hashimoto's thyroiditis, ns: not significant. ^a Dominant model (AG+GG vs. AA).

hormone profile between the TSHR genotype in GD or HT patients. All statistical analyses were performed using the SPSS 18 program (12). A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

The TSHR rs74067403 polymorphism

The PCR product is 300bp revealed on a 2% agarose gel (Fig. 1a). After digestion of the PCR fragment with *PsiI*, the wild homozygous individuals A/A show the presence of the fragments of 173 bp and 127 bp, the mutated homozygous individuals G/G present the undigested PCR product of 300bp and the heterozygous individuals A/G present fragments of 300 bp, 173 bp and 127 bp (Fig. 1b).

Genotype and allele frequencies for the TSHR rs74067403 gene in healthy controls, AITD, HT and GD patients are shown in Table 1. There was no significant difference in rs74067403 polymorphism frequencies between patients with GD and healthy control group.

The comparison of the allelic distributions showed that the mutated allele (G) is less common in AITD (31.11%), GD (31.82%) and HT (30.88%) than in controls (39.83%) but the difference is not significant. The frequency of genotypes with the mutated allele

(G) (AG + GG) is less important in patients with AITD (62.22%) and patients with HT (61.76%) than in controls, but the difference is not significant.

The TSHR rs1054708 polymorphism

The PCR product is 331 bp revealed on a 2% agarose gel (Fig. 2a). After digestion of the PCR fragment with *NlaIII*, the wild homozygous individuals T/T show the presence of the fragments of 195 bp, 80 bp and 56 bp, the mutated homozygous individuals C/C present fragments of 107 bp, 88 bp, 80 bp and 56 bp and the heterozygous individuals T/C present fragments of 195 bp, 107 bp, 88 bp, 80 bp and 56 bp (Fig. 2b).

The allele and genotype distributions of rs1054708 polymorphism in patients with AITD, GD, HT and controls are shown in Table 2. Allelic distributions comparison showed that the mutated allele (C) is less common in AITD (24.5%) and HT (20.4%) than in controls (43.2%) and this difference is highly significant ($p = 3.10^{-6}$; $p = 4.10^{-7}$ respectively).

The frequency of heterozygous genotype TC is less common in AITD (34.2%) and HT (30.6%) than in controls (41.7%) and this difference is highly significant ($p = 0.012$, $p = 0.003$ respectively).

The frequency of mutated homozygous CC genotype is less common in AITD (7.4%) and HT

Figure 2 a. Polymorphism chain reaction amplification bands for TSHR polymorphism rs1054708 (c.193335 T>C). Lane M shows DNA size marker.

Figure 2 b. Electrophoresis of enzymatic digestion for TSHR polymorphism rs1054708 (c.193335 T>C). Lane N shows DNA size marker and lane B shows negative control (no DNA).

Table 2. Distribution of rs1054708 of TSHR genotypes and alleles in patients with AITD (n=149), HT (n=98), GD (n=51) and healthy control subjects (n=134)

	Control n (%)	AITD n (%) p-value	HT n (%) p-value	GD n (%) p-value
Genotype				
TT	50 (36)	87 (58.4)	63 (64.3)	24 (47.1)
TC	58 (41.7)	51 (34.2) p=0.012, OR=0.51 CI[0.29-0.87]	30 (30.6) p=0.003, OR=0.41 CI[0.22-0.76]	21 (41.2) ns
CC	31 (22.3)	11 (7.4) p=4*10 ⁻⁵ , OR=0.2 CI[0.09-0.47]	5 (5.1) p=2*10 ⁻⁵ , OR=0.13 CI[0.04-0.38]	6 (11.7) ns
TC+CC^a	89 (64)	62 (41.6) p=2*10 ⁻⁴ , OR=0.4 CI[0.24-0.66]	35 (35.7) p=3*10 ⁻⁵ , OR=0.31 CI[0.18-0.55]	27 (52.9) ns
Allele				
T	158 (56.8)	225 (75.5)	156 (79.6)	69 (67.6)
C	120 (43.2)	73 (24.5) p=3*10 ⁻⁶ , OR=0.43 CI[0.29-0.62]	40 (20.4) p=4*10 ⁻⁷ , OR=0.34 CI[0.29-0.52]	33 (32.4) ns

AITD = autoimmune thyroid disease, GD = Graves' disease, HT = Hashimoto's thyroiditis, ns: not significant. a Dominant model (TC+CC vs. TT).

(5.1%) than in controls (22.3%) and this difference is highly significant in AITD ($p = 4.10^{-5}$; $p = 2.10^{-5}$ respectively).

The frequency of genotypes with the mutated allele (C) (TC + CC) is lower in patients with AITD (41.6%) and patients with HT (35.7%) than in controls (64%) and the difference is highly significant ($p = 2.10^{-4}$, $p = 3.10^{-5}$).

The comparison of allelic and genotypic distributions of TSHR gene in patients with Graves' disease and in controls did not show a significant difference.

Clinical characteristics and TSHR rs74067403 polymorphism

Table 3 shows the frequencies of the genotypes and alleles of the rs74067403 polymorphism as a

function of the clinical parameters of all the patients.

No association of the rs74067403 polymorphism was shown either with the sex and age of patients with AITD, HT or GD or with the presence or absence of anti-Tg and anti-TPO antibodies (Table 3).

Likewise, we did not demonstrate a significant difference in TSH and T4 levels in GD and HT patients between the rs74067403 TSHR genotypes (Table 4).

Clinical characteristics and rs1054708 polymorphism

The frequency distribution of genotypes and alleles of the polymorphism rs1045708 according to the clinical parameters of all patients with GD, HT and AITD are in Tables 5, 6 and 7 respectively.

We found no association between rs1054708

Table 3. Distribution of rs74067403 of TSHR genotypes and alleles in patients with AITD, HT and GD according to clinical parameters

	AITD						HT						GD					
	AA n (%)	AG+GG n (%)	P	A n (%)	G n (%)	P	AA n (%)	AG+GG n (%)	P	A n (%)	G n (%)	P	AA n (%)	AG+GG n (%)	P	A n (%)	G n (%)	P
Age																		
< 40	11 (32.35)	14 (25)		36 (34.61)	14 (25)		9 (34.6)	10 (23.81)		28 (29.79)	10 (23.8)		2 (25)	4 (28.57)		8 (26.67)	4 (28.57)	
≥ 40	23 (67.65)	42 (75)	ns	68 (65.39)	42 (75)	ns	17 (65.38)	32 (76.19)	ns	66 (70.21)	32 (76.2)	ns	6 (75)	10 (71.43)	ns	22 (73.33)	10 (71.43)	ns
Gender																		
F	31 (91.18)	53 (94.64)		116 (92.8)	53 (94.64)		24 (92.31)	40 (95.24)		89 (93.68)	40 (95.24)		7 (87.5)	13 (92.86)		27 (90)	13 (92.86)	
M	3 (8.82)	3 (5.36)	ns	9 (7.2)	3 (5.36)	ns	2 (7.69)	2 (4.76)	ns	6 (6.32)	2 (4.76)	ns	1 (12.5)	1 (7.14)	ns	3 (10)	1 (7.14)	ns
ATg																		
+	11 (57.89)	15 (53.57)		37 (56.06)	15 (53.57)		7 (50)	12 (54.55)		26 (52)	12 (54.55)		4 (80)	3 (50)		11 (68.75)	3 (50)	
-	8 (42.11)	13 (46.43)	ns	29 (43.94)	13 (46.43)	ns	7 (50)	10 (45.45)	ns	24 (48)	10 (45.45)	ns	1 (20)	3 (50)	ns	5 (31.25)	3 (50)	ns
ATPO																		
+	14 (77.78)	15 (53.57)		43 (67.19)	15 (53.57)		10 (76.92)	14 (63.64)		34 (70.83)	14 (63.64)		4 (80)	1 (16.67)		9 (56.25)	1 (16.67)	
-	4 (22.22)	13 (46.43)	ns	21 (32.81)	13 (46.43)	ns	3 (23.08)	8 (36.36)	ns	14 (29.17)	8 (36.36)	ns	1 (20)	5 (83.33)	ns	7 (43.75)	5 (83.33)	ns

AITD = autoimmune thyroid disease, GD = Graves' disease, HT = Hashimoto's thyroiditis, Anti-tg: anti-thyroglobulin, Anti-TPO: anti-thyroid peroxidase, F: Female, M: Male, ns: not significant.

Table 4. Hormone levels among the rs74067403 TSHR genotypes in GD and HT patients

	HT (n= 98)				GD (n= 51)			
	TSH	p-value	T4	p-value	TSH	p-value	T4	p-value
Genotype								
AA	38.04±54.4		8.28±4.64		0.33±0.77		40±26.15	
AG+GG	79.81±151.14	ns	7.37±4.58	ns	0.01±0.01	ns	58.14±57.14	ns

The range of normal values for TSH is 0.15–5 mIU/L and for ft4 is 8.6–25pmol/L. TSH, thyroid stimulating hormone; ft4, free thyroxine; HT, Hashimoto's thyroiditis; ns, not significant.

Table 5. Distribution of rs1054708 of TSHR genotypes and alleles in patients with GD according to clinical parameters

Genotypes	Sex		P	Age		P	ATg		P	ATPO		P
	F n(%)	M n(%)		< 40 n(%)	≥ 40 n(%)		- n(%)	+ n(%)		- n(%)	+ n(%)	
TT	23 (53.49)	1 (12.5)		10 (62.5)	14 (40)		4 (50)	9 (69.23)		6 (60)	7 (63.64)	
TC	15 (34.88)	6 (75)	ns	4 (25)	17 (48.57)	ns	3 (37.5)	3 (23.08)	ns	2 (20)	4 (36.36)	ns
CC	5 (11.63)	1 (12.5)	ns	2 (12.5)	4 (11.43)	ns	1 (12.5)	1 (7.69)	ns	2 (20)	0	ns
TC+CC	20 (46.51)	7 (87.5)	ns	6 (37.5)	21 (60)	ns	4 (50)	4 (30.77)	ns	4 (40)	4 (36.36)	ns
Alleles												
T	61 (70.93)	8 (50)		24 (75)	45 (64.29)		11 (68.75)	21 (80.77)		14 (70)	18 (81.82)	
C	25 (29.07)	8 (50)	ns	8 (25)	25 (35.71)	ns	5 (31.25)	5 (19.23)	ns	6 (30)	4 (18.18)	ns

Atg: anti-thyroglobulin, ATPO: anti-thyroid peroxidase, F: Female, M: Male, ns: not significant.

polymorphism and the age and sex of patients with GD and HT. No association was observed with the presence or absence of anti-thyroglobulin antibodies and anti-thyroid peroxidase antibodies.

In patients of AITD, the TC genotype is more common in men (60%) than in women (31.34%), this difference was significant ($p = 0.038$). Also this genotype is more common in patients with age greater than or equal to 40 years (38.89%) than in patients with age less than 40 years (21.95%), and this difference was

significant ($p = 0.047$). The genotypes with the mutated allele C (TC + CC) are more common among men (66.67%) than in women (38.8%), and this difference was significant ($p = 0.038$) (Table 7).

In Table 8, the serum T4 and TSH of patients with GD and HT were compared according to the rs1054708 polymorphism genotypes TSHR. We have demonstrated no significant difference in the TSH and T4 levels in patients with GD and HT between genotypes of rs1054708 polymorphism TSHR gene.

Table 6. Distribution of rs1054708 of TSHR genotypes and alleles in patients with HT according to clinical parameters

Genotypes	Sex			Age			ATg			ATPO		
	F n(%)	M n(%)	p	< 40 n(%)	≥ 40 n(%)	p	- n(%)	+ n(%)	p	- n(%)	+ n(%)	p
TT	59 (64.84)	4 (57.14)		19 (76)	44 (60.27)		20 (66.67)	17 (77.27)		13 (61.9)	22 (78.57)	
TC	27 (29.67)	3 (42.86)	ns	5 (20)	25 (34.25)	ns	8 (26.66)	5 (22.73)	ns	6 (28.58)	6 (21.43)	ns
CC	5 (5.49)	0	ns	1 (4)	4 (5.48)	ns	2 (6.67)	0	ns	2 (9.52)	0	ns
TC+CC	32 (35.16)	3 (42.86)	ns	6 (24)	29 (39.73)	ns	10 (33.33)	5 (22.73)	ns	8 (38.1)	6 (21.43)	ns
Alleles												
T	145 (79.67)	11 (78.57)		43 (86)	113 (77.4)		48 (80)	39 (88.64)		32 (76.2)	50 (89.28)	
C	37 (20.33)	3 (21.43)	ns	7 (14)	33 (22.6)	ns	12 (20)	5 (11.36)	ns	10 (23.8)	6 (10.72)	ns

Atg: anti-thyroglobulin, ATPO: anti-thyroid peroxidase, F: Female, M: Male, ns: not significant.

Table 7. Distribution of rs1054708 of TSHR genotypes and alleles in patients with AITD according to clinical parameters

Genotypes	Sex			Age			ATg			ATPO		
	F n(%)	M n(%)	p	< 40 n(%)	≥ 40 n(%)	p	- n(%)	+ n(%)	p	- n(%)	+ n(%)	p
TT	82 (61.2)	5 (33.33)		29 (70.73)	58 (53.71)		24 (63.16)	26 (74.29)		19 (61.3)	29 (74.36)	
TC	42 (31.34)	9 (60)	p=0.038 OR=3.51 CI[1.10-11.15]	9 (21.95)	42 (38.89)	p=0.047 OR=2.33 CI[1.00-5.44]	11 (28.94)	8 (22.85)	ns	8 (25.8)	10 (25.64)	ns
CC	10 (7.46)	1 (6.67)	ns	3 (7.32)	8 (7.4)	ns	3 (7.9)	1 (2.86)	ns	4 (12.9)	0	ns
TC+CC	52 (38.8)	10 (66.67)	p=0.038 OR=3.15 CI[1.02-9.74]	12 (82)	50 (46.29)	ns	14 (36.84)	9 (25.71)	ns	12 (38.7)	10 (25.64)	ns
Alleles												
T	206 (76.86)	19 (63.33)		67 (81.7)	158 (73.15)		59 (77.63)	60 (85.71)		46 (74.19)	68 (87.18)	
C	62 (23.14)	11 (36.67)	ns	15 (18.3)	58 (26.85)	ns	17 (22.37)	10 (14.29)	ns	16 (25.81)	10 (12.82)	ns

Atg: anti-thyroglobulin, ATPO: anti-thyroid peroxidase, F: Female, M: Male, ns: not significant.

Table 8. Hormone levels among the rs1054708 TSHR genotypes in GD and HT patients

Genotype	HT (n= 98)				GD (n= 51)			
	TSH	p-value	T4	p-value	TSH	p-value	T4	p-value
TT	53.923±92.709		7.878±4.562		0.739±2.154		40.02±45.233	
TC	66.597±133.258	ns	42.434±98.915	ns	0.01±0.013	ns	39.159±23.967	ns
CC	4.393±5.654	ns	10.82±6.861	ns	0.036±0.035	ns	84.007±109.193	ns

The range of normal values for TSH is 0.15–5 mIU/L and for fT4 is 8.6–25pmol/L. TSH, thyroid stimulating hormone; fT4, free thyroxine; HT, Hashimoto's thyroiditis; ns, not significant.

DISCUSSION

Given the determining role of thyroid-specific gene in AITD pathogenesis, the TSHR gene could conceivably be a candidate gene for GD and HT. In a previous study, we showed the implication of TSHR gene polymorphism D727E in HT (12) and in GD (13). This result prompted us to evaluate the susceptibility and prognostic significance of another TSHR gene polymorphism in AITD.

To our knowledge this is the first study that investigated the association of the two polymorphisms rs1054708 and rs74067403 of the TSHR gene with the development and the prognosis of AITD including

Graves' disease (GD) and Hashimoto's thyroiditis (HT).

No significant difference was found for rs74067403 TSHR polymorphism between AITD patients and healthy controls.

rs74067403 polymorphism of TSHR gene has not been associated with Graves' disease. The mutated allele G does not seem to be related to the risk of developing Graves' disease. This result is in agreement with several other studies which have studied the polymorphism D727E of TSHR gene with different populations such as Caucasian U.S. population (14, 15), the German population (16) and the Asian population (17). However, one study, conducted with the Russian

population, has shown a very strong combination ($p = 7.5 \times 10^{-6}$) of this polymorphism with Graves' disease (18).

Our study did not reveal an association of rs74067403 polymorphism with Hashimoto thyroiditis. This genetic variation does not seem to have an effect on the occurrence of this autoimmune thyroid disease. This result confirms that of Yin and collaborators in 2008. They have shown that TSHR gene may be involved in Graves' disease but not in Hashimoto thyroiditis (19). The rs74067403 polymorphism does not seem to have an effect on the occurrence of this AITD. However, we cannot exclude that another sequence of polymorphism in TSHR gene might be an important autoimmune mechanism of HT (20, 21).

For rs1054708 polymorphism, our study did not reveal an association of rs1054708 genotypes and allele with GD, and this result can be explained by the small number of GD patients. On the other hand, we found an association between HT and AITD and heterozygous genotype TC, the mutated genotype CC and the genotypes with mutated allele C (TC+CC). These genotypes are associated with HT and with AITD as protective factor ($OR < 1$). The mutated allele C is also associated with HT and with AITD as protective factor ($OR = 0.34$, $OR = 0.43$ respectively). Our results suggest that the TSHR rs1054708 polymorphism can be a protective factor against HT and AITD.

Therefore, the functional effects of this polymorphism on TSHR production remain to be evaluated in AITD Tunisian patients.

By the analysis of our results according to the severity and prognosis of AITD (HT and GD), we have not shown a relationship between rs1054708 and rs74067403 polymorphisms of TSHR gene and the presence of anti TPO and anti Tg antibodies in patients with GD and HT suggesting that humoral relations in the thyroid can be independent to TSHR gene polymorphism.

On the other hand we investigate the association of these two polymorphisms of TSHR and age and gender of patients.

The heterozygous genotype TC and genotypes with the mutated allele (TC+CC) of the rs1054708 polymorphism are associated with male patients of AITD. The heterozygous genotype TC is associated with age of onset of disease. These results suggest that the rs1054708 polymorphism can affect the etiology of AITD between men and women also by age.

We were unable to find any significant

association between rs1045708 and rs74067403 polymorphisms and change in the serum concentrations of TSH and FT4 in AITD patients. Nevertheless previous studies have found an association between another polymorphism of TSHR gene (D727E polymorphism) and reduced serum TSH levels (22-24).

Our study has several limitations which must be clarified. The interaction between genetic factors and various environmental factors could affect the mechanism of development of thyroid autoimmune diseases which are complex and have multi-factorial pathology, which is not easily explained by case-control studies. In addition, we need to increase the number of patients and controls as well as their clinical data to improve confidence in our results. Other functional studies are also necessary to better understand this pathology.

In conclusion, this study examined the prevalence of TSHR genotype polymorphisms and their association with AITD and with clinical parameters. These preliminary results suggest that TSHR rs74067403 polymorphism may be a risk factor for late onset of AITD and can affect the etiology of AITD between men and women. Nevertheless the rs1054708 polymorphism may be associated with the protection against AITD.

Conflict of interest

The authors declare that they have no conflict of interest. No funding was allocated for this study.

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