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ORIGINAL ARTICLE

Gender-dependent and age-of-onset-specific association of the rs11675434 single-nucleotide polymorphism near *TPO* with susceptibility to Graves' ophthalmopathy

Aleksander Kuś[1], Konrad Szymański[2], Beata Jurecka-Lubieniecka[3], Edyta Pawlak-Adamska[4], Dorota Kula[3], Piotr Miśkiewicz[1], Marek Bolanowski[5], Rafał Płoski[2], Artur Bossowski[6], Jacek Daroszewski[5], Barbara Jarząb[3] and Tomasz Bednarczuk[1]

The role of *TPO* gene polymorphism in the susceptibility to Graves' disease (GD) remains unclear. However, single-nucleotide polymorphisms (SNPs) near *TPO* have been recently associated with serum levels of thyroid peroxidase (TPO) antibody in two independent genome-wide association studies. Moreover, we have observed a strong association between the rs11675434 SNP located near *TPO* and the presence of clinically evident Graves' ophthalmopathy (GO). The aim of the current study was to reevaluate and dissect this association in an extended group of 1231 well-characterized patients with GD (1043 adults and 188 children) and 1130 healthy controls from the Polish Caucasian population, considering possible gender-dependent and age-of-onset-specific effects of the studied SNP. We found that the T allele of rs11675434 was significantly more frequent in GD patients with than without GO (odds ratio (OR) = 1.26, 95% confidence interval (CI) = 1.05–1.51, *P* = 0.012), which was consistent with our previous findings. Further analyses performed in subgroups of patients showed that the association with GO was significant in adult patients with age of GD onset ⩾45 years (OR = 1.34, 95% CI = 1.03–1.75, *P* = 0.031), but not in children and adolescents or adult patients with earlier onset of the disease (OR = 1.72, 95% CI = 0.77–3.84, *P* = 0.18 and OR = 1.05, 95% CI = 0.79–1.40, *P* = 0.75, respectively). Moreover, a strong association with GO was present in males (OR = 2.06, 95% CI = 1.40–3.02, *P* = 0.0002), whereas it was absent in females (OR = 1.10, 95% CI = 0.90–1.35, *P* = 0.35). The results of our study further suggest that rs11675434 SNP located near TPO is associated with the development of GO, especially in males and patients with later age of GD onset.
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INTRODUCTION

The thyroid peroxidase (TPO) is a key enzyme of thyroid function, having a pivotal role in the synthesis of thyroid hormones. Whereas multiple studies showed that various *TPO* gene mutations may cause dysfunction of the TPO enzyme in patients with congenital hypothyroidism,[1,2] the role of *TPO* gene polymorphisms in the susceptibility to autoimmune thyroid disease remains unclear. Interestingly, single-nucleotide polymorphisms (SNPs) near *TPO* have been recently associated with TPO antibody (TPOAb) serum levels in two independent population-based genome-wide association studies conducted in Caucasian and Asian populations.[3,4] As the appearance of TPOAb often precedes the development of an overt autoimmune thyroid disease, including Graves' disease (GD), we assessed the association of the most significantly associated SNP in the *TPO* region identified in the genome-wide association study by Medici *et al.*[3] (rs11675434) with susceptibility to and phenotype of GD in a cohort of GD patients from the Polish population.[5] Although rs11675434 was not associated with susceptibility to GD, we observed a strong association between this SNP and the presence of clinically evident Graves' ophthalmopathy (GO).[5] Taking into consideration the emerging evidence for gender-dependent differences in effects of genetic polymorphisms on the regulation of thyroid function,[6] and gene–environment interactions in patients with late and early onset of the disease resulting from differing lengths of exposure to

[1]Department of Internal Medicine and Endocrinology, Medical University of Warsaw, Warsaw, Poland; [2]Department of Medical Genetics, Centre for Biostructure, Medical University of Warsaw, Warsaw, Poland; [3]Department of Nuclear Medicine and Endocrine Oncology, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland; [4]Department of Experimental Therapy, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland; [5]Department of Endocrinology, Diabetes and Isotope Therapy, Wroclaw Medical University, Wroclaw, Poland and [6]Department of Pediatrics, Endocrinology and Diabetes with a Cardiology Unit, Medical University of Bialystok, Bialystok, Poland
Correspondence: Professor T Bednarczuk, Department of Internal Medicine and Endocrinology, Medical University of Warsaw, ul. Banacha 1a, 02-097 Warsaw, Poland.
E-mail: tomasz.bednarczuk@wum.edu.pl
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environmental factors,[7,8] the aim of the current study was to reevaluate the association between rs11675434 and GO in an extended group of well-characterized patients with GD, as well as to assess the differences in gender-dependent and age-of-onset-specific effects of the studied SNP.

MATERIALS AND METHODS

Subjects

A total of 1231 GD patients from the Polish Caucasian population, including 1043 adults (827 females, 216 males) and 188 unrelated children and adolescents (149 females, 39 males) with a confirmed diagnosis of GD were included in the study. The adult patients were recruited from three independent cohorts: Warsaw, Gliwice and Wroclaw, whereas children and adolescent patients were recruited mainly from the Bialystok cohort; all cohorts have been described in previous studies.[8–11] The diagnosis of GD was established on the basis of clinical and biochemical symptoms of hyperthyroidism accompanied by diffuse goiter, detectable TSH receptor autoantibodies and/or increased radioiodine uptake, as described in the previous studies.[8–11] The severity of the GO was assessed using NOSPECS classification,[12] and clinically evident GO was defined as: proptosis, extraocular muscle dysfunction, exposure keratitis and optic neuropathy (NOSPECS $\geqslant 3$, moderate-to-severe and sight-threatening ophthalmopathy basing on EUGOGO consensus[13]). The clinical characteristics of the study group are shown in Table 1.

The control group consisted of 970 healthy adults (730 females, 240 males) and 160 unrelated healthy children and adolescents (75 females, 85 males), and comprised cohorts used in the previous studies.[8–10]

Written informed consent was collected from all of the participants and the local ethical committees of the participating institutions approved the study.

Genotyping

The analyzed SNP was genotyped using a real-time PCR method with a predesigned TaqMan SNP Genotyping Assay provided by Applied Biosystems, Woolston, Warrington, UK. The genotyping procedure was performed according to the manufacturer's protocol on the QuantStudioTM 12K Flex instrument (Life Technologies Corporation, Carlsbad, CA, USA). The overall genotyping call rate was 96.65%.

Statistical analysis

$P<0.05$ was considered statistically significant. The genotype distributions of the analyzed SNP were tested for Hardy–Weinberg equilibrium and showed no evidence of deviation ($P=0.80$ and $P=0.77$ in the total group of patients and controls, respectively). The χ^2-test showed no significant difference in the genotype distributions within the analyzed cohorts of GD patients ($P=0.42$) and they were analyzed together.

Allele frequencies and genotype distributions in the whole group of GD patients and controls were compared using the χ^2-test. The genotype distributions were analyzed assuming different models of inheritance (dominant, additive and recessive). The association with susceptibility to GD was also analyzed separately for subgroups of GD patients stratified on the basis of gender (males, females) and age of GD onset (children and adolescents with GD onset $\leqslant 18$ years, younger adults with GD onset 18–45 years and older adults with GD onset $\geqslant 45$ years).

A similar comparison of allele frequencies and genotype distributions with subgroup analysis was subsequently performed between GD patients with clinically evident GO (NOSPECS class 3–6) and without clinically evident GO (NOSPECS class 0–1), as well as healthy controls. As GD patients with a milder phenotype of ophthalmopathy, characterized by involvement of only the soft tissue (NOSPECS class 2), tend to be variously classified in different studies, which has a negative impact on the reliability of the results obtained, they were excluded from the current analyses.

Web-Assotest (http://www.ekstroem.com/assotest/assotest.html) and Statistica (StatSoft, Tulsa, OK, USA) software were used for the analyses.

RESULTS

Association with GD

In the allele analysis, rs11675434 showed no association with susceptibility to GD in the total group of analyzed subjects (odds ratio (OR) = 1.09, 95% confidence interval (CI) = 0.97–1.22, $P=0.16$). Neither further subgroup analyses revealed any gender-dependent or age-of-onset-specific effects of the studied SNP on GD susceptibility (Table 2). The study had power (alpha = 0.05, power = 0.8) to detect allelic OR = 1.18, considering alleles prevalence in the control group (minor allele frequency, (MAF) = 0.41) and the total number of subjects analyzed. Likewise, there was no significant difference in the

Table 1 Clinical characteristics of the study group

	Warsaw (N = *647*)		*Gliwice* (N = *196*)		*Wroclaw* (N = *256*)		*Bialystok* (N = *132*)		*Total* (N = *1231*)	
Characteristics	N_{av}	n *(%)*	N_{av}	n *(%)*	N_{av}	n *(%)*	N_{av}	n *(%)*	N_{av}	n *(%)*
Male	647	130 (20.1)	196	35 (17.9)	256	56 (21.9)	132	34 (25.8)	1231	255 (20.7)
Age of onset in years (mean ± s.d.)	609	39.98 ± 14.78	196	44.15 ± 13.20	255	46.38 ± 11.83	132	13.69 ± 2.93	1192	39.12 ± 16.07
Disease duration in years (mean ± s.d.)	609	4.78 ± 6.09	185	9.76 ± 5.65	255	3.25 ± 4.22	128	2.75 ± 3.27	1177	5.01 ± 5.83
Ophthalmopathy (NOSPECS class)	603		193		256		132		1184	
Class 0		309 (51.2)		85 (44.0)		63 (24.6)		132 (100.0)		589 (49.7)
Class I		41 (6.8)		21 (10.9)		2 (0.8)		0 (0.0)		64 (5.4)
Class II		38 (6.3)		28 (14.5)		66 (25.8)		0 (0.0)		132 (11.1)
Class III		105 (17.4)		42 (21.8)		47 (18.4)		0 (0.0)		194 (16.4)
Class IV		89 (14.8)		17 (8.8)		53 (20.7)		0 (0.0)		159 (13.4)
Class V		8 (1.3)		0 (0.0)		23 (9.0)		0 (0.0)		31 (2.6)
Class VI		13 (2.2)		0 (0.0)		2 (0.8)		0 (0.0)		15 (1.3)
Cigarette smokers	587	263 (44.8)	179	81 (45.3)	256	106 (41.4)	132	0 (0.0)	1154	450 (39.0)
Family history of AITD	562	169 (30.1)	191	58 (30.4)	253	61 (24.1)	132	28 (21.2)	1138	316 (27.8)
Therapy for hyperthyroidism	600		196		253		132		1181	
Antithyroid drugs only		196 (32.7)		6 (3.1)		127 (49.6)		132 (100.0)		461 (39.0)
Radioactive iodine		319 (53.2)		192 (97.9)		25 (9.8)		0 (0.0)		536 (45.4)
Surgery		86 (14.3)		17 (8.7)		101 (39.5)		0 (0.0)		204 (17.3)

Abbreviation: AITD, autoimmune thyroid disease; N_{av}, number of subjects available for analysis.

genotype distribution at rs11675434 between the total group of GD patients and controls, nor in any subgroup of GD patients analyzed separately (Supplementary Table 1).

Association with GO

On the contrary, the T allele was significantly more frequent in patients with than without clinically evident GO (OR = 1.26, 95% CI = 1.05–1.51, $P = 0.012$), which was consistent with our previous findings.[5] Further analyses performed in subgroups of patients showed that the association with GO was borderline significant in older adults (OR = 1.34, 95% CI = 1.03–1.75, $P = 0.031$), but not in children and adolescents (OR = 1.72, 95% CI = 0.77–3.84, $P = 0.18$) or in younger adults (OR = 1.05, 95% CI = 0.79–1.40, $P = 0.75$). Moreover, a strong association was present in males (OR = 2.06, 95% CI = 1.40–3.02, $P = 0.0002$), whereas it was absent in females (OR = 1.10, 95% CI = 0.90–1.35, $P = 0.35$; Table 3). Similarly, the genotype distributions at rs11675434 were significantly different in GD patients with and without GO in the total group of patients, whereas in subgroup analysis, a significant difference was observed in males and older adults (Supplementary Table 2). We did not find any significant difference in the allele/genotype distribution at rs11675434 between patients with moderate-to-severe and sight-threatening ophthalmopathy (data not shown). When we compared subsets of GO patients with healthy controls, we confirmed a significant difference in allele distributions in the total group of GO patients (Table 4, OR = 1.31, 95% CI = 1.11–1.54, $P = 0.0014$), as well as in GO subsets of males and adult patients with later age of GD onset (OR = 1.58, 95% CI = 1.17–2.15, $P = 0.0031$ and OR = 1.39, 95% CI = 1.13–1.70, $P = 0.0017$, respectively). Although we also observed a borderline significant difference in females (OR = 1.23, 95% CI = 1.03–1.48, $P = 0.024$), this association was absent when we compared subsets of female GD patients with and without GO. The differences in genotype distributions at rs11675434 between GO patients and controls are

Table 2 A comparison of the rs11675434 allele frequencies in the control group, total group of GD patients and subgroups of GD patients stratified by gender and age of GD onset

		Allele C		*Allele T*			
Group	N_{av}	n	%	n	%	*OR (95% CI)*	P-*value*
Controls	1084	1283	59.18	885	40.82	Reference	
GD total	1198	1369	57.14	1027	42.86	1.09 (0.97–1.22)	0.16
GD males	252	296	58.73	208	41.27	1.02 (0.84–1.24)	0.85
GD females	946	1073	56.71	819	43.29	1.11 (0.98–1.25)	0.11
GD older adults	508	572	56.30	444	43.70	1.13 (0.97–1.31)	0.12
GD younger adults	471	541	57.43	401	42.57	1.07 (0.92–1.25)	0.36
GD children and adolescents	182	212	58.24	152	41.76	1.04 (0.83–1.30)	0.74

Abbreviations: CI, confidence interval; GD, Graves' disease; N_{av}, number of subjects available for analysis; OR, odds ratio.

Table 4 A comparison of the rs11675434 allele frequencies in the control group, total group of GD patients with clinically evident GO (NOSPECS class 3–6), and subgroups of GO patients stratified by gender and age of GD onset

		Allele C		*Allele T*			
Group	N_{av}	n	%	n	%	*OR (95% CI)*	P-*value*
Controls	1084	1283	59.18	885	40.82	Reference	
GO total	388	408	52.58	368	47.42	1.31 (1.11–1.54)	0.0014
GO males	90	86	47.78	94	52.22	1.58 (1.17–2.15)	0.0031
GO females	298	322	54.03	274	45.97	1.23 (1.03–1.48)	0.024
GO older adults	223	228	51.12	218	48.88	1.39 (1.13–1.70)	0.0017
GO younger adults	149	166	55.70	132	44.30	1.15 (0.90–1.47)	0.25
GO children and adolescents	13	12	46.15	14	53.85	1.69 (0.78–3.67)	0.18

Abbreviations: CI, confidence interval; GO, Graves' ophthalmopathy; N_{av}, number of subjects available for analysis; OR, odds ratio.

Table 3 A comparison of the rs11675434 allele frequencies between GD patients with clinically evident GO (NOSPECS class 3–6) and without clinically evident GO (NOSPECS class 0–1) in the total group of GD patients and in subgroups of GD patients stratified by gender and age of GD onset

			Allele C		*Allele T*			
Group	*NOSPECS class*	N_{av}	n	%	n	%	*OR (95% CI)*	P-*value*
GD total	0–1	640	746	58.28	534	41.72	**1.26 (1.05–1.51)**	**0.012**
	3–6	388	408	52.58	368	47.42		
GD males	0–1	134	175	65.30	93	34.70	**2.06 (1.40–3.02)**	**0.0002**
	3–6	90	86	47.78	94	52.22		
GD females	0–1	506	571	56.42	441	43.58	1.10 (0.90–1.35)	0.35
	3–6	298	322	54.03	274	45.97		
GD older adults	0–1	215	251	58.37	179	41.63	**1.34 (1.03–1.75)**	**0.031**
	3–6	223	228	51.12	218	48.88		
GD younger adults	0–1	256	291	56.84	221	43.16	1.05 (0.79–1.40)	0.75
	3–6	149	166	55.70	132	44.30		
GD children and adolescents	0–1	161	192	59.63	130	40.37	1.72 (0.77–3.84)	0.18
	3–6	13	12	46.15	14	53.85		

Abbreviations: CI, confidence interval; GD, Graves' disease; N_{av}, number of subjects available for analysis; OR, odds ratio. Bold entries indicate significant *P*-values ($P < 0.05$).

shown in Supplementary Table 3. Importantly, if a conservative Bonferroni's correction adjusted for a number of tests performed was applied, the only significant association between the studied SNP and GO in our study was found between male GD patients with and without GO ($P = 0.0002$, $P_{cor} = 0.014$).

DISCUSSION

The results of our study further suggest that rs11675434 SNP located near *TPO* may be associated with the development of clinically evident GO, especially in males and patients with later age of GD onset.

Being located on the apical membrane of thyrocytes, the TPO enzyme is responsible for the iodination of the tyrosine residues in thyroglobulin, and coupling of the iodinated tyrosines to generate triiodothyronine (T3) and thyroxine (T4).[14] It is a large glycoprotein encoded by the *TPO* gene located on chromosome 2, consisting of over 150 kb and 17 exons.[15] rs11675434 is a SNP located in the upstream region of the *TPO* gene (NBCI build 36, chr2:1386822). Kwak *et al.*[4] showed that rs2071403, a SNP in linkage disequilibrium with rs11675434 ($r^2 = 0.509$, $D' = 1.00$ in CEU population), is associated with the TPOAb serum levels, as well as with the TPO messenger RNA levels in human thyroid tissue. Several studies suggested that TPOAb may take part in the antibody-dependent thyrotoxicity in autoimmune thyroid disease patients in both cell-mediated and complement-dependent mechanisms.[16,17] Interestingly, the *TPO* gene expression in retrobulbar tissue in both GD patients and healthy subjects have been reported.[18] Although a predominant role of the autoantibodies against the thyroid-stimulating hormone receptor (TRAb) in the pathogenesis of GO seems to be established,[19–21] the TPOAb-dependent cytotoxicity mechanisms may be also involved in the process, potentially explaining the presence of GO symptoms in TRAb-negative patients. However, the association between TPOAb and GO is still questionable, as the results of previous studies on this issue are highly inconsistent. Whereas it was reported that the lower TPOAb serum levels are associated with an increased risk of GO development in GD patients,[22] other studies yielded the opposite results[23] or negated the association.[24] Therefore further studies on the pathogenesis of GO are needed to clarify the role of TPOAb, and explain the underlying mechanisms of the observed association between rs11675434 and GO. As ocular complications may significantly reduce the quality of life in GD patients,[25] identifying ones with high risk of developing severe ophthalmopathy before the complications occur could also allow clinicians to provide patients with an early, adjusted and thus more effective treatment.

Some interesting findings of our study are the gender-dependent and age-of-onset-specific differences in the observed effects of the studied SNP. Although their underlying mechanisms remain unclear, these results correspond with the gender-dependent differences in effects of genetic polymorphisms on the regulation of thyroid function reported recently by Porcu *et al.*[6] Also the results of our recent research on the association between the *TSHR* gene polymorphism and the susceptibility to GO suggested a significant association in patients with early onset of GD, which was not observed in patients with later onset of the disease, nor in the total group of GD patients analyzed in the study.[26] Whereas twin studies clearly indicate that the genetic predisposition has the major role in GD,[27] still only ~10% of the genetic susceptibility can be explained based on the loci identified in previous studies, including genome-wide association studies.[28] Therefore, improved phenotyping and use of precise phenotypes in genetic association studies may provide a better insight into the genetic background of the disease.[29] As the results of our study support that hypothesis, we strongly recommend to include genotype–phenotype association analyses in further studies. On the other hand, multiple testing used for subsets analyses may increase the risk of false-positive findings. If a conservative Bonferroni's correction was applied, the association between the studied SNP and GO in our study remained significant in male patients only. Therefore, our results require confirmation in other cohorts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)