Title

Common genetic variants associated with thyroid function may be risk alleles for Hashimoto's disease and Graves' disease.

Short title

Risk alleles for Hashimoto's disease and Graves' disease.

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Summary

Background Recent studies have identified common genetic variants associated with TSH, free T4 and thyroid peroxidase antibodies, but it is unclear if these differ between patients with Hashimoto's disease and Graves' disease.

Objective To examine whether 11 common genetic variants differ between Graves' disease and Hashimoto's disease.

Patients and measurements We genotyped 11 common variants in a discovery cohort of 203 Australian patients with autoimmune thyroid disease (AITD). Two variants with significant or suggestive associations were analysed in a replication cohort of 384 Danish patients.

Results For rs753760 (*PDE10A*), the minor allele frequency in Graves' disease and Hashimoto's disease was 0.38 vs. 0.23 respectively ($P=6.42 \times 10^{-4}$) in the discovery cohort, 0.29 vs. 0.24 ($P=0.147$) in the replication cohort and 0.32 vs. 0.24 in combined analysis ($P=0.0021$; all analyses adjusted for sex). In healthy controls from Busselton, the frequency was 0.29, significantly different from Hashimoto's disease but not Graves' disease. For rs4889009 (*MAF* gene region), the frequency of the minor G allele in Graves' disease and Hashimoto's disease was 0.48 vs. 0.36 ($P=0.0156$) in the discovery cohort, 0.48 vs. 0.34 ($P=1.83 \times 10^{-4}$) in the replication cohort and 0.48 vs. 0.35 in the combined analysis ($P=7.53 \times 10^{-6}$); in controls the frequency was 0.38, significantly different from Graves' disease but not Hashimoto's disease. After further adjustment for smoking, associations with rs4889009 remained significant, whereas those with rs753760 were not.

Conclusion Common variants in *PDE10A* and *MAF* gene regions may influence whether patients with AITD develop Graves' disease or Hashimoto's disease.

Introduction

The thyroid is targeted by autoimmune responses more frequently than any other organ (1). Clinically, the most common presentations of autoimmune thyroid disease (AITD) are hypothyroidism caused by Hashimoto's disease and Graves' hyperthyroidism. Both diseases are characterised by lymphocytic infiltration of the thyroid and the production of thyroid autoantibodies (1-3). In Graves' disease, thyrotoxicosis results from the production of stimulating antibodies to the TSH receptor, (1), whereas Hashimoto's disease is characterised by tissue destruction and consequent hypothyroidism (4). Circulating thyroid peroxidase antibodies (TPOAb) are detectable in approximately 90-95% of patients with Hashimoto's disease and 75-85% of those with Graves' disease (5), whereas antibodies against the sodium iodide symporter (NIS) and pendrin are very rare, and absent in healthy controls (6). In contrast, TPOAb are also present in about 10% of the general population (7, 8), but most TPOAb-positive individuals do not develop clinically overt thyroid disease.

The factors which trigger an autoimmune response to the thyroid and influence its progression to clinical thyroid disease are only partly understood (1, 9, 10). Environmental factors implicated in AITD include iodine status, infection, and tobacco smoking; the latter appears to increase the risk of Graves' disease while being protective against Hashimoto's disease (11). Familial clustering of AITD is common, and in twin studies, its heritability is approximately 70%, indicating a strong genetic influence (10, 12). Over the past two decades, considerable progress has been made in identifying susceptibility genes for AITD (1, 10, 13-15). These include a range of immunoregulatory genes (*HLA-DRB1*, *CTLA-4*, *PTPN22*, *ARID5B*, *FCRL3*, *CD40*, *CD25*, *IL12B*), many of which are also associated with other autoimmune diseases, and a smaller number of thyroid-specific genes (*TSHR*, *TG*). In a study published in 2011, however, known susceptibility genes for Graves' disease accounted for less than 10% of the heritability of this disease in a Chinese Han population (16). In a recent meta-analysis of genome wide association (GWA) studies, Medici et al. (17) reported 5 novel loci associated with TPOAb: common variants in *TPO*, *ATXN2* and *BACH2* were associated with TPOAb-positivity, whereas variants in *TPO*, *MAGI3* and *KALRN* were associated with TPOAb concentrations.

Most AITD susceptibility genes identified to date are not specific to Graves' disease or Hashimoto's disease, with the exception of *TSHR*, *CD40* and *CD25*, which are associated with Graves' disease. We previously reported that an *IL12B* polymorphism differed between Graves' disease and Hashimoto's disease in men but not women with AITD (18). In the study by Medici et al. (17), the *BACH2* variant was associated with hyperthyroidism but not hypothyroidism and with Graves' disease in an independent cohort, suggesting that it may be specific to Graves' disease, whereas the *MAGI3* variant was associated with both hyperthyroidism and hypothyroidism.

TSH and free T4 are also heritable traits (19, 20), and recent studies have advanced understanding of their genetic architecture (21-24). In a recent meta-analysis of GWA studies, Porcu et al. (22) identified common variants in 26 loci associated with TSH or free T4 in euthyroid individuals. Several loci, including *PDE8B*, *PDE10A* and *CAPZB* were also associated with elevated serum TSH concentrations, suggesting a possible role in hypothyroidism.

In view of the very limited evidence regarding genetic risk factors for Hashimoto's disease versus Graves' disease, we compared the genotype frequencies of common variants in 11 genes identified from the two recent meta-analyses in participants with these disorders from two well-defined clinical cohorts.

Methods

Participants were Caucasian patients diagnosed with Graves' disease (defined as hyperthyroidism accompanied by positive TSH receptor antibodies and/or diffuse tracer uptake at thyroid scintigraphy) or Hashimoto's disease (defined as spontaneously occurring hypothyroidism accompanied by elevated TPOAb concentration and/or pathologically demonstrated lymphocytic thyroiditis). The discovery cohort was recruited from outpatient clinics at Sir Charles Gairdner Hospital in metropolitan Perth, Western Australia, whereas the replication cohort comprised clinic patients from Odense University Hospital, Odense, Denmark as previously described (18). The aim of the study was to compare genotypes between Graves' disease and Hashimoto's disease, and for that reason the study protocol did not include recruitment of a

control group free of thyroid disease. However, for genotypes which differed significantly between Graves' disease and Hashimoto's disease, we compared our results with genotypes from 3960 healthy controls (2145 women and 1815 males) from the Busselton Health Study using genome wide association study data. Clinical data and genotyping methods for the Busselton study have been published previously (17, 24). Healthy controls were defined as TPOAb-negative with TSH levels between 0.4 and 4.0 mU/L and no history of thyroid disease.

In the discovery cohort, genotyping was performed on DNA extracted from venous blood of each participant for 11 variants, one each per gene as follows: *TPO* (rs11675434), *ATXN2* (rs653178), *BACH2* (rs10944479), *MAGI3* (rs1230666), *KALRN* (rs2010099), *MAF* (rs4889009), *PDE10A* (rs753760), *PDE8B* (rs1382879), *CAPZB* (rs10799824), *VEGFA* (rs9472138) and *FOXE1* (rs7848973). Five of these genes (*TPO*, *ATXN2*, *BACH2*, *MAGI3*, *KALRN*) were associated with TPOAb in the meta-analysis by Medici et al. (22); of the remainder, 5 were associated with TSH (*MAF*, *PDE10A*, *PDE8B*, *CAPZB*, *VEGFA*) and 1 with free T4 (*FOXE1*) in the meta-analysis by Porcu et al. (22). In addition, rs4889009 (*MAF*) showed suggestive association with TPOAb-positivity in the study by Medici et al. (17) but not genome-wide significance. Genotyping was performed using TaqMan allelic discrimination 5'nuclease assays (Applied Biosystems, Foster City, CA) in a reaction volume of 5 ul containing assay-specific primers and allele-specific probes according to the manufacturer's protocol. Fluorescence was measured by a Victor2 Multilabel Plate-Reader (Perkin-Elmer, Waltham, MA). Two variants, rs4889009 and rs753760, showed significant or suggestive associations in the discovery cohort and were analysed in the replication cohort using the same techniques.

In the primary analysis, binary association tests were performed using general linear modelling in R version 3.1.0 and SNPTTEST v2.3.0, adjusting for sex. Results were very similar for the two methods, and those from SNPTTEST are presented. Correction for multiple testing was performed using the Bonferroni method, with significance set at $P < 0.0045$ ($0.05/11$ variants). In a secondary analysis, we further analysed for smoking habits (current, former, never), because of the recognised association between smoking and AITD phenotype (11). In the discovery cohort, smoking data was not recorded in the original study (18); for the

present study, we contacted participants to obtain these data, which were available from 50% of the cohort; the rest of the cohort was not included in this analysis. Smoking data was available for all members of the replication cohort. General linear modelling was performed to determine whether smoking was associated with AITD.

Written informed consent was obtained from participants. Ethical approval was obtained from Human Research Ethics Committees at Sir Charles Gairdner Hospital and Odense University Hospital.

Results

The discovery cohort comprised 203 Australian patients with AITD (104 with Hashimoto's disease and 99 with Graves' disease) and the replication cohort 384 Danish patients (169 with Hashimoto's disease and 215 with Graves' disease). Descriptive data are shown in Table 1. Smoking habit differed significantly between Graves' disease and Hashimoto's disease using Chi squared test ($P=2.48 \times 10^{-9}$).

In the discovery cohort, 9 of the 11 variants analysed showed no significant difference in genotype frequency between Hashimoto's disease and Graves' disease ($P>0.4$ for each). For rs753760 in *PDE10A*, there was a significant difference between groups ($P=6.42 \times 10^{-4}$) (Table 2) after adjustment for sex, whereas for rs4889009 in the *MAF* region, after Bonferroni correction there was only suggestive evidence of a difference ($P=0.0156$). In the replication cohort, genotypes for rs753760 in *PDE10A* did not differ significantly between groups ($P=0.147$), whereas for rs4889009 in the *MAF* gene region there was a significant difference after Bonferroni correction ($P=1.83 \times 10^{-4}$).

When results from both cohorts were combined, there was a significant difference in genotype frequency between Graves' disease and Hashimoto's disease for both variants. For rs753760 in *PDE10A*, the minor allele frequency was 0.24 for Hashimoto's disease and 0.32 for Graves' disease ($P=0.0023$) with each additional copy of the minor allele (G) associated with a 33% decrease in the odds of an individual having Hashimoto's disease versus Graves' disease. In healthy controls free of thyroid disease, the minor allele

frequency was 0.29; this was significantly different from Hashimoto's disease ($P=8.21 \times 10^{-3}$) but not from Graves' disease ($P=0.173$). For rs4889009 in the *MAF* gene region, the minor allele frequencies were 0.35 for Hashimoto's disease and 0.48 for Graves' disease in the combined analysis ($P=7.53 \times 10^{-6}$), with each additional copy of the minor allele associated with a 68% increase in the odds of having Graves' disease versus Hashimoto's disease. The frequency of the minor G allele for rs4889009 in healthy controls was 0.38; this was significantly different from Graves' disease ($P=1.01 \times 10^{-5}$) but not from Hashimoto's disease ($P=0.053$).

Smoking data were available for 50% of the discovery cohort and all of the replication cohort. After adjustment for sex and smoking habits, the difference in genotype frequency between Graves' disease and Hashimoto's disease in the combined analysis was no longer significant for rs753760 in *PDE10A* (minor allele frequency 0.30 vs. 0.24, $P=0.0957$) but remained significant for rs4889009 in the *MAF* region (0.48 vs. 0.35, $P=4.05 \times 10^{-4}$) (Table 3).

Discussion

In this study, we examined common variants in 11 genes known to be associated with TPOAb, TSH or free T4 to ascertain whether their genotype frequency differed between Hashimoto's disease and Graves' disease. For two variants, there was strong or suggestive evidence of a difference between groups in the discovery and replication cohort, and in both cases the difference was significant after adjustment for sex in the combined analysis. The first of these was the single nucleotide polymorphism (SNP) rs753760, located in intron 1 of *PDE10A*, for which the minor allele (G) was less frequent in participants with Hashimoto's disease compared with Graves' disease and with healthy controls, suggesting that the major allele (C) may predispose to Hashimoto's disease. rs753760 is reported by Haploreg v3 (25) to alter a regulatory motif in MEF2C. MEF2C, among other functions, plays key roles in B lymphocyte formation and function, including antibody responses to T-cell-dependent antigens and induction of germinal centre B cells (26). The SNP is also in strong linkage disequilibrium (LD) ($r^2 \geq 0.8$) with 15 genetic variants located at chr6:166040859-166060601 (hg19), but none of these has functional annotation that appears relevant to AITD. rs753760 has

not been previously associated with thyroid autoimmunity, and it is possible that in patients with AITD, it influences whether the phenotype is Hashimoto's disease or Graves' disease. *PDE10A* encodes a cAMP-stimulated phosphodiesterase which is implicated in cAMP degradation in response to TSH stimulation of thyrocytes (22, 27), and in the meta-analysis by Porcu et al. (22), the major C allele was positively associated with TSH in euthyroid participants, suggesting a physiological rather than pathological role. It is plausible, therefore that the variant influences disease ascertainment rather than pathogenesis, since individuals with thyroid autoimmunity who have the C allele will be more likely to have an elevated TSH level than those who do not, and are therefore more likely to have Hashimoto's disease diagnosed.

The second variant which differed between groups was rs4889009, located at chromosome 16q23, for which the minor allele was associated with an increased risk of Graves' disease compared with Hashimoto's disease and compared with healthy controls. In the study of Medici et al. (17), there was suggestive evidence that this SNP was associated with TPOAb-positivity, but the association was not genome-wide significant. rs4889009 is an intergenic variant, reported by Haploreg v3 to be located in a weak enhancer region of chromatin regulatory states in mobilized CD34 primary cells. It is in strong LD ($r^2 \geq 0.8$) with 45 other variants located chr16:79696939-79734249, none of which has functional annotation that seems relevant to AITD. The SNP is also in moderate LD ($r^2=0.686$ for each) with two variants: rs17767491, which is associated with thyroid volume (28) and rs3813582, associated with serum TSH (22), whereas rs4889009 itself is not known not to be associated with TSH. In this region lies a third variant, rs3813579, associated with goitre (28) but this is in low LD with rs4889009 ($r^2=0.222$). The three variants lie within 4kb of each other and 110kb upstream from *MAF*, and rs17767491 and rs3813579 were found not to be in LD with *MAF* (28). However, these variants do encompass the 3'-end of *LOC440389* plus the region immediately downstream. *LOC440389* is a predicted coding sequence previously removed from the NCBI database as a result of standard genome annotation processing. Teumer et al. (28) have shown that sequenced cDNA from mRNA extracted from thyroid tissue matches the published *LOC440389* mRNA sequence, suggesting that the removal of *LOC440389* from the NCBI database may have been inappropriate. Teumer et al. (28) also found this transcript to be threefold more abundant in thyroid versus skeletal muscle tissue, providing tentative

evidence that *LOC440389* is a thyroid-specific gene, but its function is unknown. That locus also maps within *LOC102467146*, a large non-coding RNA. Our data suggest that it may be a susceptibility locus for Graves' disease.

In the present study, participants with Graves' disease were significantly more likely to be current smokers than those with Hashimoto's disease, consistent with previous reports (11, 29). When the results were adjusted for smoking as well as sex, the associations with rs4889009 (*MAF*) remained significant, but results for rs753760 (*PDE10A*) were no longer significant. As information on smoking habits were not available for all participants, this may reflect a loss of statistical power as well as the confounding effect of smoking. Not all studies of genetic associations with AITD have adjusted for smoking, despite its association with AITD phenotype, and our study demonstrates the importance of this.

For the remaining 9 variants studied, including 5 associated with TPOAb in the recent meta-analysis (17), there was no significant difference between Hashimoto's disease and Graves' disease. Since TPOAb are associated with both Hashimoto's disease and Graves' disease, this may be because these variants are risk factors for AITD *per se* rather than a specific AITD phenotype; alternatively it may be that our study was too small to detect genuine differences between Hashimoto's disease and Graves' disease because of the relatively small effect size of those risk alleles. Our study was adequately powered to detect variants with clinically relevant effect sizes; for example for rs4889009 with a minor allele frequency of 0.38 associated with a 68% increase in the odds of having Graves' disease versus Hashimoto's disease per copy of the G-allele, the study had 93% power to detect an association at $\alpha=0.05$ (30). However, for a genetic variant with the same minor allele frequency but a lower effect size such as a 50% increase per copy of the risk allele, the power was slightly less: 77% at $\alpha=0.05$.

Strengths of our study include the carefully defined phenotypes of Hashimoto's disease and Graves' disease established in specialist clinics. A limitation of our study is that we did not have smoking data from all participants in the discovery cohort, which reduced the power of the smoking-adjusted analyses.

In conclusion, this study provides evidence that common variants in the *PDE10A* and *MAF/LOC440389* gene regions differ between patients with Hashimoto's disease and Graves' disease. These findings advance understanding of the genetic architecture and pathophysiology of AITD. Further studies are required to establish the mechanisms underlying these associations, and the extent to which rare variants (as opposed to common variants as studied here) contribute to the heritability and phenotype of AITD. In addition, our study provides additional evidence for a role for *LOC440389* in thyroid physiology, strengthening the case for its reinstatement to the NCBI database and for further research to determine the function of this gene and its role in thyroid biology.

Table 1: Characteristics of the discovery cohort and replication cohorts. Data shown as number and percentage.

	Perth	Odense
<u>Graves' disease</u>	99	215
Males	14 (14%)	34 (16%)
Females	85 (86%)	181 (84%)
Smoking*	49	215
Never	20 (41%)	73 (34%)
Former	18 (37%)	33 (15%)
Current	11 (22%)	109 (51%)
<u>Hashimoto's disease</u>	104	169
Males	10 (10%)	26 (15%)
Females	94 (90%)	143 (85%)
Smoking*	52	169
Never	28 (54%)	92 (54%)
Former	18 (35%)	43 (25%)
Current	6 (12%)	34 (20%)

* Data available for 101/203 (50%) of participants in the Perth cohort. For smoking data in Hashimoto's disease, percentages do not add up to 100% because of rounding.

Table 2: Genotyping results for rs753760 (*PDE10A*) and rs4889009 (*MAF*) in participants with Graves' disease versus Hashimoto's disease phenotype using SNPTTEST adjusting for sex.

	Graves' disease					Hashimoto's disease							
	N	1.1	1.2	2.2	MF	N	1.1	1.2	2.2	MF	P	β	SE
<i>rs753760</i>													
Perth	99	38	47	14	0.38	104	59	42	3	0.23	6.42x10⁻⁴	-0.76	0.22
Odense	213	106	92	15	0.29	169	101	55	13	0.24	0.147	-0.24	0.16
Combined	312	144	139	29	0.32	273	160	97	16	0.24	0.002	-0.40	0.13
<i>rs4889009</i>													
Perth	96	24	52	20	0.48	104	46	42	16	0.36	0.016	-0.49	0.20
Odense	214	65	94	55	0.48	169	75	74	20	0.34	1.83x10⁻⁴	-0.53	0.14
Combined	310	89	146	75	0.48	273	121	116	36	0.35	7.53x10⁻⁶	-0.52	0.12

Data shown as number of subjects. For rs753760 allele 1 = C and allele 2 = G; for rs4889009 allele 1 = C and allele 2 = G. MF, minor allele frequency. For Perth cohort, 3 individuals failed genotyping for rs7889009 and for Odense cohort, 1 person failed genotyping for rs753760.

Table 3: Genotyping results for rs753760 (*PDE10A*) and rs4889009 (*MAF*) in the participants with Graves' disease versus Hashimoto's disease phenotype using SNPTTEST adjusting for sex and smoking.

	Graves' disease					Hashimoto's disease					P	β	SE
	N	1.1	1.2	2.2	MF	N	1.1	1.2	2.2	MF			
rs753760													
Perth	49	21	23	5	0.34	52	28	22	2	0.25	0.140	-0.50	0.34
Odense	213	106	92	15	0.29	169	101	55	13	0.24	0.214	-0.22	0.17
Combined	262	127	115	20	0.30	221	129	77	15	0.24	0.096	-0.26	0.15
rs4889009													
Perth	49	12	29	8	0.46	52	22	20	10	0.38	0.211	-0.37	0.30
Odense	214	65	94	55	0.48	169	75	74	20	0.34	8.18x10 ⁻⁴	-0.50	0.15
Combined	263	77	123	63	0.47	221	97	94	30	0.35	4.05x10 ⁻⁴	-0.47	0.13

Data shown as number of subjects. For rs753760 allele 1 = C and allele 2 = G; for rs4889009 allele 1 = C and allele 2 = G. MF, minor allele frequency. For Odense cohort, 1 person failed genotyping for rs753760.

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