Genetic association studies of the *FOXP3* gene in Graves' disease and autoimmune Addison's disease in the United Kingdom population

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Abstract

Regulatory T lymphocytes play a crucial role in modulating potentially self-reactive clones, and dysfunction of this cell type contributes to autoimmune disease. *FOXP3* is a critical determinant of CD⁴⁺CD²⁵⁺T regulatory (T_{reg}) cell development and function. The aim of this study was to investigate whether genetic polymorphisms at the *FOXP3* locus predispose to autoimmune endocrinopathies. Five single nucleotide polymorphisms (SNPs) and two microsatellite polymorphisms were genotyped in our Caucasian cohorts of 633 unrelated Graves' disease (GD) subjects, 104 autoimmune Addison's disease (AAD) subjects and 528 healthy controls. SNP genotyping was performed by either restriction enzyme digestion or by primer-extension-MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) assay. Microsatellites were analysed using fluorescent PCR. Case-control analysis was performed using χ^2 testing on contingency tables for allele frequency. Haplotype analysis was performed using the UNPHASED package. No evidence for disease association was found with any of the seven polymorphisms in either of the GD or AAD subjects as compared with controls ($P=0.26-0.94$). Haplotype analysis found a weak evidence for the association of a minor haplotype with GD; this was not significant when corrected for multiple testing. This study has found no robust evidence that *FOXP3* gene polymorphism contributes to the susceptibility to GD or AAD in the UK population.

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Introduction

Autoimmune endocrinopathies, including Graves' disease (GD) and autoimmune Addison's disease (AAD), are the commonest type of autoimmunity in Western populations. However, their pathogenesis remains poorly defined. Like many other autoimmune disorders, they are thought to result from a failure of the normal immune tolerance mechanisms, which includes pathogenic T cells directed against self-antigens. CD⁴⁺CD²⁵⁺T regulatory cells (T_{reg}) have a role in the modulation of potentially self-reactive T-cell clones, as they actively suppress the immune responses of autoreactive T cells (Maloy & Powrie 2001, Sakaguchi *et al.* 2001). A lack of regulatory T cells in mice caused by mutation of the *FOXP3* gene leads to severe organ-specific autoimmunity (Brunkow *et al.* 2001). Recessive X-linked mutations in the *FOXP3* gene have also been found to be responsible for the immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) in humans (Chatila *et al.* 2000, Bennett *et al.* 2001b, Wildin *et al.* 2001). This is a rare and devastating lymphoproliferative condition of male infants resulting in multiple autoimmune disorders,

in particular enteropathy, diabetes mellitus, haemolytic anaemia and thyroid autoimmunity (Wildin *et al.* 2002). Several mutations distributed throughout the *FOXP3* gene have been shown to be responsible for many cases of IPEX (Bennett *et al.* 2001a, Kobayashi *et al.* 2001, Wildin *et al.* 2002, Gambineri *et al.* 2003, Owen *et al.* 2003). Rare monogenic phenotypes like this can be helpful in identifying genes, which may also contribute to the commoner complex genetic conditions. *FOXP3* is a forkhead/winged helix transcription factor, which has been shown to be specifically expressed in naturally arising regulatory T cells, and can convert naïve T cells to this regulatory phenotype (Fontenot *et al.* 2003, Hori *et al.* 2003, Khatri *et al.* 2003, Walker *et al.* 2003a). Thus, *FOXP3* is a critical regulator of regulatory T-cell development and function. It has been shown that expression of *FOXP3* in CD⁴⁺T cells correlates with their ability to function as regulatory T cells (Walker *et al.* 2003b).

Although IPEX represents an extreme autoimmune phenotype, reduced circulating numbers of regulatory T cells have been reported in the commoner conditions such as type 1 diabetes (T1D), multiple sclerosis and autoimmune polyglandular syndrome type II (Kukreja

et al. 2002, Kriegel *et al.* 2004, Huan *et al.* 2005). One of these studies showed that the impaired regulatory T-cell function was linked to abnormalities in FOXP3 message and protein expression levels within the cells (Huan *et al.* 2005). Thus, structural, quantitative or regulatory polymorphism at the FOXP3 locus may be contributing to the susceptibility of these commoner conditions. Thus, FOXP3 is a good candidate gene to play a role in organ-specific autoimmune diseases, in particular diabetes mellitus and thyroid autoimmunity, in view of their prominence in the IPEX syndrome.

As with many other autoimmune disorders, there is a marked gender bias in both GD and AAD prevalence, with a female to male ratio of at least 5:1 in GD (Tunbridge *et al.* 1977) and 3:1 in AAD (Laureti *et al.* 1999). However, the reason for this is unclear. One explanation could be related to genes residing on the sex chromosomes, which in the setting of a complex inheritance pattern may alter the risk for the development of autoimmunity (Barbesino *et al.* 1998, Lockshin 2002). Recently, skewed X-inactivation has been demonstrated in autoimmune thyroid disease and other forms of autoimmunity (Ozbalkan *et al.* 2005, Ozelik *et al.* 2006). Furthermore, it has been hypothesised that skewed X-inactivation in the thymus may lead to inadequate thymic deletion of certain X-linked self-antigens (Stewart 1998, Chitnis *et al.* 2000). Two genome-wide scans for linkage in GD have identified putative X-chromosome loci, Xq21 (Tomer *et al.* 1999) and Xp11 (Taylor *et al.* 2006), and these loci have also been identified in localised linkage scans of the X-chromosome, Xq21 (Barbesino *et al.* 1998) and Xp11 (Imrie *et al.* 2001). However, several other genome-wide linkage scans in GD in a variety of different populations have found no evidence of linkage to the X-chromosome (Sakai *et al.* 2001, Allen *et al.* 2003, Jin *et al.* 2003, Tomer *et al.* 2003). Xp11 has also been linked to other autoimmune disorders: T1D, multiple sclerosis and rheumatoid arthritis, suggesting the presence of common susceptibility polymorphism(s) (Ebers *et al.* 1996, Cornelis *et al.* 1998, Cucca *et al.* 1998). The FOXP3 gene is located at Xp11.23 within this area of autoimmune disease linkage, thus strengthening its position as a putative susceptibility gene in autoimmune disease, particularly disorders showing a female predominance.

Bassuny and colleagues (2003) reported an association of a functional microsatellite polymorphism, (GT)*n*, located in the promoter/enhancer region of FOXP3, with T1D in a Japanese population. Subsequently, a detailed and comprehensive haplotype survey of five FOXP3 polymorphisms including the (GT)*n* promoter microsatellite was carried out in a large T1D cohort (Zavattari *et al.* 2004). However, this study did not replicate the previous findings, showing no evidence of an association between FOXP3 and T1D

(Zavattari *et al.* 2004). The role of FOXP3 in other autoimmune diseases, particularly inflammatory bowel disease, has also recently been investigated, again with discrepant results (Park *et al.* 2005, Sanchez *et al.* 2005).

We hypothesise that FOXP3 may be a stronger candidate gene for the autoimmune disorders, which in contrast to T1D, exhibit a marked female predominance. Therefore, the aim of this study was to examine whether polymorphisms in the FOXP3 gene may contribute to the susceptibility to GD and AAD in our United Kingdom (UK) cohort of unrelated patients.

Materials and methods

Subjects

Blood samples were obtained from 633 GD probands, of which 495 (78.2%) were female, and 104 AAD probands, of which 73 (70.2%) were female. Patients were all of Caucasian origin from the northeast of England, and were recruited through endocrine and thyroid eye clinics at the Newcastle upon Tyne Hospitals Trust and surrounding district hospitals. GD was defined by the biochemical presence of hyperthyroidism together with one of either a diffuse increase in thyroid uptake on ⁹⁹Tc radionuclide scanning, positive thyroid autoantibodies (thyroid peroxidase (TPO) or thyroid binding inhibiting immunoglobulin (TBII)) or thyroid-associated ophthalmopathy (NOSPECS class 3 or worse) (Werner 1977). All AAD probands had biochemical evidence of adrenal failure and infective and infiltrative causes were excluded, as were patients with Acebutolol et Prevention Secondaire de l'Infarctus (APS1).

Totally 528 controls, of which 350 (66.3%) were female, were recruited from the same local population to determine background population allele frequencies. All were Caucasian and had parents born in the northeast of England. They had no clinical features or family history of autoimmune disease.

All the studies were approved by the Newcastle and North Tyneside ethics committee and all subjects gave informed consent.

Selection of polymorphisms in the FOXP3 gene

Seven polymorphisms (five single nucleotide polymorphisms (SNPs) and two microsatellites) were selected for genotyping in our patient and control cohorts (Fig. 1). These spanned 15.7 kb of the FOXP3 region, with three being promoter SNPs, two intronic and two located in the 3'UTR. The SNPs were selected to extract the most genetic information based on marker frequency (minor allele frequency (MAF) >5%) and haplotype data using the HAPMAP database (<http://www.hapmap.org>) (Altshuler *et al.* 2005) and the

Figure 1 Schematic structure of the 12 exons (one of which, -1, is non-coding) of the *FOXP3* gene. Exons are depicted by black boxes and non-coding regions as white boxes. The position of the five SNPs and two microsatellites spanning 15.7 kb of the *FOXP3* region are shown by the arrows. The SNP ID, polymorphism, position in relation to the transcription start site (kb) and the pairwise linkage disequilibrium (r^2) measures between the seven polymorphisms are detailed below. The white boxes represent the lowest r^2 values, while darker the shading the higher the r^2 value between the relevant markers.

HAPLOVIEW program (Barrett *et al.* 2005). No exonic SNPs were included as there are no informative exonic SNPs reported in the HAPMAP and other SNP databases. The five SNPs selected covered the common SNP haplotype blocks in the *FOXP3* gene, i.e. the haplotype 'tag' SNPs, rs3761549, rs2280883, rs2294021 and rs6609857. One further SNP from the Zavattari *et al.* (2004) study was also included, rs2232365. This study identified a set of SNPs, which extracted the most genetic information from the *FOXP3* gene and the rs2232365 SNP was the only one of these SNPs that was not included in the HAPMAP analysis of the *FOXP3* gene. The two microsatellites typed by Bassuny and colleagues (2003) were also genotyped, in view of their finding of the association of these microsatellites with T1D.

Single nucleotide polymorphism genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the Nucleon BACC2 kit (Amersham Pharmacia Biotech). Genotyping was performed by PCR and restriction enzyme digest (RFLP) for one of the SNPs (rs3761549) and by primer-extension-

MALDI-TOF assay (Sequenom, San Diego, CA, USA) for the other four SNPs. Details of the primers and reaction conditions are given in Table 1.

Genotyping of (GT)*n* and (TC)*n* microsatellite polymorphisms

PCR primer pairs, sequences and nomenclature as described previously (Bassuny *et al.* 2003) were used to amplify intron zero containing the (GT)*n* microsatellite polymorphism (located in IVS0 from -150 to -177, up to -187) and intron five containing the (TC)*n* microsatellite polymorphism (located in IVS5 from +476 to +595, up to +539). The 5' end of each of the forward primers was labelled with a fluorescent dye, 6-carboxyfluorescein (FAM) dye for the (GT)*n* forward primer, and 6-carboxy-2',4,7,7'-tetrachloro-fluorescein (TET) dye for the (TC)*n* forward primer. Genotyping was performed in a mixture of both (GT)*n* and (TC)*n* amplified products, together with a 400 bp size standard (Amersham Rox ET400) by the MegaBACE sequencing instrument (Amersham Pharmacia Biotech). Results were analysed using the MegaBACE genetic profiler software (Amersham). In

Table 1 Details of the primers and enzymes used for the PCR/RFLP genotyping assay, and the primers and termination mix for the SNPs genotyped by MALDI-TOF mass spectrometry (Sequenom). Multiplex assays were designed using the MassARRAY assay design software program (Sequenom)

	Nucleotide change	Forward primer	Reverse primer	Restriction enzyme
dbSNP ID				
RFLP/PCR genotyping				
rs3761549	C/T	CTGAGACTTTGGGACCG-TAG	TGCGCCGGGCTTCATC-GACA	BseN1
SEQUENOM genotyping				
	Primer 1	Primer 2	Extension primer	Termination mix
rs2232365	ACGTTGGATGAGGCTT-GAGGGCCAGAAATT	ACGTTGGATGTGG-CAAGTGACAGAGAGGAG	GCCCCAGCTCAAGA-GACCCC	ACT
rs2280883	GTTGGATGAGCCTT-TATGCCAACCCAA	ACGTTGGAT-GATGGGTGTTACAAG-GAAAGG	ACCCCCAACTGGGCACC	ACT
rs2294021	ACGTTGGATGAGACAA-GATCTGGCAGACAC	ACGTTGGATGTACACAT-GAGGACCCCTCCAC	AGATCTGGCAGACAC-CATGGC	ACT
rs6609857	ACGTTGGATGATACA-GAGGTGGACAGAGAG	ACGTTGGATGTGTGTT-CACCCAGGTATCAG	AGGAAGAGGGGACAGAT	ACT

order to confirm the repeat number of polymorphisms for PCR product length, alleles from six males of each of the two commonest genotypes for each polymorphism (24 alleles in total) were subject to direct DNA sequencing using the MegaBACE sequencing instrument.

Statistical analysis

The case-control association studies were analysed using χ^2 tests on 2×2, 2×6 or 2×20 contingency tables for allele frequencies depending upon allele number. Haplotype frequencies were estimated using the UNPHASED package (Dudbridge 2003). Odds ratios and confidence intervals were calculated using Woolf’s method (Woolf 1995). Observed and expected genotypes in female samples were compared using the Hardy–Weinberg equation. This study had 80% power to detect an effect with an odds ratio of 1.25 for the most informative SNP rs2280883 ($\alpha=0.05$), and >90% power to detect an effect with an odds ratio of 1.5 for all the SNPs ($\alpha=0.05$), given the actual control allele frequencies found.

Results

The seven polymorphisms were genotyped in a total of 1128 GD chromosomes, 177 AAD chromosomes and 878 control chromosomes. The allele frequency data, together with odds ratios are shown in Table 2. In a marker-by-marker analysis using χ^2 testing, there was no significant difference in allele frequency and thus no evidence for disease association with any of the seven polymorphisms in either GD or AAD probands

as compared to controls was found (Table 2). Analysis of each polymorphism in the subset of 244 GD subjects with thyroid-associated ophthalmopathy vs controls also showed no significant difference in allele frequency (data not shown). Haplotype analysis was carried out on the five SNP haplotypes as shown in Table 3. One of these haplotypes ‘CATTC’, showed weak association in the GD patients with a χ^2 of 6.05, uncorrected $P=0.01$ and an odds ratio of 1.51 (1.09–2.11, 95% confidence interval (C.I.)). However, this difference did not reach a statistical significance after a Bonferroni multiple adjustment for the 15 possible haplotypes was seen.

Haplotype analysis of all seven markers was also carried out. Five common haplotypes (frequency >0.01 in all the three groups) were identified. Taking into account the number of haplotype permutations seen ($n>20$), none of these showed an association after correcting for multiple testing.

Discussion

This study has found no robust evidence for the *FOXP3* gene contributing to the susceptibility to GD or AAD in our northeast England population. This is in contrast to the original study by Bassuny *et al.* (2003), who found a significantly higher frequency of the (GT)₁₅ allele of the microsatellite (GT)_n marker in Japanese patients with T1D compared with controls. They went on to demonstrate a significant difference in the enhancer activity of the (GT)₁₅ compared with the (GT)₁₆ allele in a dual-luciferase reporter assay. Zavattari *et al.* (2004) were unable to replicate this association when they examined five polymorphisms in a Sardinian T1D population, and thus concluded that

Table 2 Allele frequencies for the seven *FOXP3* polymorphisms in controls, GD and AAD together with case-control analysis of the alleles in each disease group in comparison with controls

	Control chromosomes <i>n</i> (%)	GD chromosomes <i>n</i> (%)	Odds ratio (95% C.I.)	<i>P</i> value	AAD chromosomes <i>n</i> (%)	Odds ratio (95% C.I.)	<i>P</i> value
Marker							
rs3761549							
C	682 (85.7)	977 (87.3)	1.15	0.30	149 (84.2)	1.12	0.26
T	114 (14.3)	142 (12.7)	(0.87–1.51)		28 (15.8)	(0.70–1.80)	
Total	796	1119			177		
rs2232365							
G	489 (62.5)	675 (60.9)	1.07	0.50	113 (64.2)	1.07	0.66
A	293 (37.5)	433 (39.1)	(0.88–1.30)		63 (35.8)	(0.75–1.53)	
Total	782	1108			176		
(GT) <i>n</i>							
3	492 (56.1)	516 (52.9)	1.14 (0.95–1.38)	0.27	107 (60.5)	0.84 (0.59–1.18)	0.77
4	333 (38.0)	396 (40.6)	0.90 (0.74–1.09)		62 (35.0)	1.14 (0.80–1.62)	
5	46 (5.3)	59 (6.1)	0.86 (0.57–1.30)		8 (4.5)	1.17 (0.52–2.74)	
Other	5 (0.6)	4 (0.4)	–		0	–	
Total	876	975			177		
(TC) <i>n</i>							
4	532 (60.8)	565 (57.7)	1.14 (0.94–1.37)	0.42	114 (64.4)	0.86 (0.60–1.22)	0.32
12	62 (7.1)	87 (8.9)	0.78 (0.55–1.11)		7 (4.0)	1.85 (0.80–4.50)	
14	126 (14.4)	145 (14.8)	0.97 (0.74–1.26)		23 (13.0)	1.13 (0.68–1.87)	
Other	155 (17.7)	182 (18.6)	–		33 (18.6)	–	
Total	875	979			177		
rs2280883							
T	418 (53.6)	591 (53.4)	1.01 (0.83–1.22)	0.91	91 (51.7)	1.08 (0.77–1.52)	0.64
C	362 (46.4)	516 (46.6)			85 (48.3)		
Total	780	1107			176		
rs2294021							
C	484 (62.3)	676 (60.4)	1.08 (0.89–1.31)	0.41	113 (64.6)	1.10 (0.77–1.58)	0.57
T	293 (37.7)	443 (39.6)			62 (35.4)		
Total	777	1119			175		
rs6609857							
C	566 (72.9)	821 (72.8)	1.01 (0.82–1.24)	0.94	133 (76)	1.17 (0.79–1.75)	0.41
T	210 (27.1)	307 (27.2)			42 (24)		
Total	776	1128			175		

C.I., confidence interval.

Table 3 Analysis of the five marker (SNPs only) and seven marker (including microsatellites) haplotypes. The five SNP haplotypes are made up of the alleles: rs3761549–rs2232365–rs2280883–rs2294021–rs6609857 respectively. The seven-marker haplotype also includes the two microsatellite markers (GT)*n* and (TC)*n*. Only haplotypes with frequencies >0.01 in each group are listed

	Controls	GD	<i>P</i> value	OR (95% C.I.)	AAD	<i>P</i> value	OR (95% C.I.)
Five marker halotype							
CGCCC	0.50	0.45	0.07	0.83 (0.68–1.02)	0.47	0.64	0.92 (0.64–1.32)
CATTT	0.25	0.27	0.36	1.11 (0.89–1.40)	0.25	0.95	1.01 (0.67–1.54)
TGTCC	0.14	0.14	0.70	1.06 (0.79–1.41)	0.16	0.51	1.18 (0.72–1.96)
CATTC	0.09	0.13	0.01	1.51 (1.09–2.11)	0.11	0.47	1.25 (0.69–2.27)
Other	0.02	0.01	–	–	0.01	–	–
Seven marker halotype							
CGCCC 3 4	0.59	0.48	0.01	0.65 (0.49–0.87)	0.52	0.22	0.77 (0.49–1.21)
TGTCC 3 4	0.15	0.18	0.17	1.26 (0.84–1.89)	0.19	0.25	1.39 (0.76–2.53)
CATTC 4 14	0.08	0.10	0.24	1.30 (0.77–2.20)	0.10	0.56	1.21 (0.54–2.68)
CATTT 4 14	0.03	0.03	0.92	1.05 (0.41–2.59)	0.07	0.08	0.43 (0.14–1.30)
CGCCC 4 4	0.02	0.03	0.48	1.31 (0.50–3.57)	0.03	0.53	1.42 (0.33–5.66)
Other	0.13	0.18	–	–	0.09	–	–

OR, odds ratio.

variation at or near *FOXP3* is not associated with T1D. There have also been discrepant reports on the role of *FOXP3* in non-endocrine autoimmune diseases (Park *et al.* 2005, Sanchez *et al.* 2005). A true association of an allele with a disease would not be expected to be positive in every study population, and this may be compounded by the different ethnic origins of the sample populations in the three studies of autoimmune endocrinopathies to date. The initial positive study (Bassuny *et al.* 2003) was carried out in a Japanese population whilst the two negative studies were both in white Caucasians (Sardinian (Zavattari *et al.* 2004) and United Kingdom (UK) populations (current study)). Recent reports have shown a significant difference in allele frequencies among different ethnicities (Yamazaki *et al.* 2004, Mori *et al.* 2005), particularly between Japanese and Caucasian populations (Yamazaki *et al.* 2004), and our results illustrate this. The allele frequencies of the markers in common between the studies is similar in the two European populations (UK and Sardinian), but markedly different within the Japanese population (25% difference in allele frequency for marker (GT) $_n$ allele 4). This ethnic variation in disease susceptibility alleles has been seen recently with other genes, e.g. PADI4 gene polymorphisms have been associated with rheumatoid arthritis in the Japanese population (Suzuki *et al.* 2003) but not within European populations (Barton *et al.* 2004, Caponi *et al.* 2005, Martinez *et al.* 2005).

A meta-analysis summary of common disease susceptibility polymorphisms in different populations found that for a replicated disease-associated allele, the relative risk tended to be similar in different ethnic groups despite different background allele frequencies (Ioannidis *et al.* 2004). It is clearly the case that, if a true susceptibility locus has been identified, then an association should be found in every population. However, if the candidate allele is only in linkage disequilibrium with the true susceptibility locus, the association may only exist in certain populations due to ancestral haplotype structure (Gambano *et al.* 2000). This may lead to the rejection of a true association in a specific population because the allele being typed is not the actual disease susceptibility locus. Inconsistencies between different ethnic groups may also reflect complex interactions between multiple population-specific genetic and environmental factors.

In summary, we have found no robust evidence for association of the *FOXP3* gene with GD or AAD in our northeast of England population. However, we cannot rule out the possibility that a polymorphism carried on the minor haplotype (CATTC) that shows nominal evidence for association ($P=0.01$) might have a role. Nevertheless, we have gleaned genetic information

encompassing more than 98% of all haplotype combinations across the 15.7 kb *FOXP3* locus by genotyping these five SNPs and two microsatellites (Table 2). *FOXP3* is a candidate gene for autoimmune diseases mainly due to its increasingly understood important role in immune regulation and the fact that mutations in the gene are responsible for the condition IPEX. However, more specific studies into the functional role of this gene in autoimmune disease will be necessary before it can be said to have a biologically defined role in autoimmunity. There is still no clear answer as to whether *FOXP3* is relevant in more complex autoimmune endocrinopathies such as T1D, GD and AAD, and further studies on large cohorts of patients with these conditions in a variety of ethnic populations are still required to increase our knowledge-base for this gene.

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