

**GENOMIC POLYMORPHISM AT THE INTERFERON-INDUCED HELICASE
(IFIH1) LOCUS CONTRIBUTES TO GRAVES' DISEASE SUSCEPTIBILITY**

Alison Sutherland,¹ Jocelyn Davies,¹ Catherine J Owen,^{1,2} Suresh Vaikkakara,³ Christine Walker,² Timothy D Cheetham,^{2,3} R Andrew James,^{2,3} Petros Perros,^{2,4} Peter T Donaldson,² Heather J Cordell,¹ Richard Quinton,^{2,3} Simon HS Pearce.^{1,2,3}

Institute of Human Genetics¹ and School of Clinical Medical Sciences,² Newcastle University; and Endocrine Unit, Royal Victoria Infirmary,³ and Freeman Hospital,⁴ Newcastle upon Tyne, UK.

Address for Correspondence:

Dr. Simon Pearce,
Institute of Human Genetics, Newcastle University,
International Centre for Life, Central Parkway,
Newcastle upon Tyne, NE1 3BZ, United Kingdom.
Tel. 44-191-241-8674 Fax. 44-191-241-8666

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ABSTRACT

Context: A recent large-scale analysis of non-synonymous coding polymorphisms showed strong evidence that an alanine to threonine amino acid change at codon 946 of the interferon-induced helicase (*IFIH1*) gene (SNP ID rs1990760) was associated with type 1 diabetes. Previous investigations have also demonstrated that an intronic polymorphism (termed *PDI.3*; SNP ID rs11568821) in the programmed cell death (*PDCDI*) gene was associated with systemic lupus erythematosus and rheumatoid arthritis.

Objective: We sought to replicate these genetic associations in Graves' disease and autoimmune Addison's disease patient cohorts.

Patients and Methods: Six hundred and two Graves' disease subjects, 214 Addison's disease subjects and 446 healthy controls were genotyped for the *IFIH1* and *PDCDI* single nucleotide polymorphisms using mass spectrometer analysis of primer extension products (Sequenom).

Results: The alanine carrying allele at the *IFIH1* codon 946 polymorphism was present in 796 of 1204 (66%) GD patient alleles compared to 508 of 892 (57%) control subject alleles; odds ratio 1.47 (5-95% CI 1.23 to 1.76), $p=1.9 \times 10^{-5}$. In contrast, there was no association of alleles at this marker in autoimmune Addison's disease. Neither was there evidence for association in either patient cohort at the *PDI.3* polymorphism.

Conclusions: We confirm a significant contribution of the Ala946Thr *IFIH1* polymorphism to organ-specific autoimmune diseases, extending the range of conditions associated with this variant to include Graves' disease. This polymorphism may also contribute to several other autoimmune disorders.

INTRODUCTION

There is a substantial contribution of genetic factors to the pathogenesis of autoimmune endocrine disorders (1,2), with one twin study suggesting that more than three-quarters of the predisposition to Graves' disease (GD, autoimmune hyperthyroidism) is determined by heredity (3). Genomic loci that make a significant and consistent contribution to autoimmune thyroid disease (AITD) susceptibility include the Major Histocompatibility Complex, the cytotoxic T lymphocyte antigen-4 locus and an allele at the protein tyrosine phosphatase non-receptor 22 gene (encoding the lymphoid tyrosine phosphatase 620W variant) (1,2,4,5). These alleles have also been shown, in varying degrees, to have a role in type 1 diabetes (T1D), autoimmune Addison's disease (AAD), rheumatoid arthritis and several other autoimmune conditions, demonstrating that several loci may be considered as common autoimmunity loci (1,2,5), presumably with a general predisposing effect to "skew" the immune response towards autoreactivity. These loci stand in contrast to disease specific loci, which tend to have a tissue-specific expression pattern, such as the insulin gene variable number tandem repeat (VNTR) polymorphism in T1D and the TSH-receptor in GD (6-8).

Recently, using preliminary results from large-scale association analysis of non-synonymous single nucleotide polymorphisms (SNPs), a novel locus for T1D was identified, the interferon-induced helicase (*IFIH1*), also known as the melanoma differentiation-associated 5 (MDA-5) or *Helicard* gene (9). The *IFIH1* gene encodes a viral RNA-activated apoptosis protein, with a putative role in sensing and triggering clearance responses in virally infected cells (10,11). Using a logistic regression analysis,

the most associated marker in *IFIH1* was defined as the SNP rs1990760, which encodes an alanine to threonine amino acid change at codon 946, with an odds ratio for association with T1D of 1.16 (5-95% confidence interval 1.11- 1.22) for the major allele (9). Another molecule with a role in cellular apoptosis pathways that has been demonstrated to have a role in autoimmunity is the cell-surface programmed cell death 1 receptor, encoded by the *PDCDI* gene. Several large studies have shown association of polymorphisms in *PDCDI* with systemic lupus erythematosus and rheumatoid arthritis (12-14). The most associated variant (designated PD1.3 or rs11568821) lies within intron 4 of the gene and has been demonstrated to alter a RUNX1 transcription factor binding site rendering RUNX1 unable to bind to the *PDCDI* sequences (12). Both the *IFIH1* and *PDCDI* genes encode transcripts that have widespread expression in lymphoid and other tissues, suggesting that they could have a role in many autoimmune conditions. In this study we have examined the *IFIH1* Ala946Thr, and the PD1.3 SNPs in cohorts of Caucasians with GD and AAD to determine whether genomic polymorphism at these apoptosis-related loci may have a wider role in autoimmune endocrinopathy.

METHODS

Materials and Methods

Subjects

The GD (n=602) probands were recruited through endocrine and thyroid associated ophthalmopathy clinics in Newcastle and the surrounding district hospitals. Of the Addison's disease probands 105 were recruited from endocrine clinics in the North East of England, with a further 109 recruited via meetings of the UK Addison's disease self-help group (ADSHG). The diagnostic criteria for GD were biochemical hyperthyroidism with confirmation by either serum autoantibodies, radionuclide scan or presence of ophthalmopathy (15). Of the GD probands 78% were female, 40% had significant thyroid associated ophthalmopathy (defined as NOSPECS class 3 or worse) and 55% were cigarette smokers. The Northeast AAD patients had a serum cortisol of less than 550nmol/l following ACTH stimulation, with a raised basal plasma ACTH level, or pigmentation. Patients with tuberculosis, those with another known non-autoimmune cause, and those with autoimmune hypoparathyroidism or candidiasis (subjects with type 1 polyendocrinopathy) were excluded from the cohort (16). AAD subjects recruited from ADSHG were all interviewed by CW or SHSP. A self-reported diagnosis of AAD in subjects with an appropriate medical history who were currently taking hydrocortisone and fludrocortisone was taken as evidence of AAD. The median age of onset, male to female ratio and prevalence of other autoimmune conditions was not different between AAD subjects recruited from our hospital services and from ADSHG. The AAD cohort included 161 females (75.2%) and 53 males (24.8%). Isolated AAD accounted for 36% of the AAD cohort, the other 64% had at least one other associated autoimmune disease

(hypothyroidism, 80; Graves' disease, 25; primary gonadal failure, 24; type 1 diabetes, 12; pernicious anaemia, 14; vitiligo, 6; celiac disease, 6; rheumatoid arthritis, 4; alopecia, 3; haemolytic anaemia, 2; and autoimmune hepatitis, 1). UK controls (n=446; 66.3% females, 33.7% males) also recruited from the local population had no clinical features or family history of autoimmune disease.

SNP genotyping

Both the rs1990760 SNP in IFIH1 and the rs11568821 (PD1.3) SNP were genotyped using MALDI-TOF mass spectrometry of primer extension products (Sequenom iPLEX system, Hamburg, Germany). Amplification primers, including tag sequences were as follows: rs1990760- ACGTTGGATGCTCCATGATGATTCTTTCCC and ACGTTGGATGTTTGATAGTCGGCACACTTC; rs11568821- ACGTTGGATGAGGCAGGCACACACATGGG and ACGTTGGATGCAACCTCAATCCCTAAAGCC. Extension primers were as follows: rs1990760- TTTACATTGTAAGAGAAAACAAA; rs11568821- CATTGGCCGGGCACCCCGGAGAC. All reactions were carried out in a final volume of 5µl according to manufacturer's conditions. Typing of ten percent of the samples was repeated blind to confirm assay fidelity.

Statistical analysis

The case-control association studies were analysed using χ^2 tests on 2x2 and 2x3 contingency tables for allele and genotype frequencies, respectively. Odds ratios and confidence intervals were calculated using Woolf's method. Heterogeneity between the odds ratios in our study and those from the published analysis of T1D (9) was examined

using an interaction term in a logistic regression analysis. No significant deviation from Hardy Weinberg equilibrium was observed for either of the SNPs in this study (all $p > 0.05$). We estimated that the power of our GD study was $>90\%$, assuming an allelic odds ratio of 1.3 and an allele frequency of 0.40, as previously determined in UK controls at rs1990760 ($\alpha = 0.05$) (9). Assuming an OR of 1.16 (as previously found in T1D), we estimate that more than 900 probands and matched controls would be needed to give 80% power ($\alpha = 0.05$).

RESULTS

Both alleles and genotypes of the IFIH1 Ala946Thr (rs1990670) SNP were significantly associated with GD, with the Ala carrying allele being present in 796 of 1204 (66%) GD patient alleles compared to 508 of 892 (57%) control subject alleles; odds ratio 1.47 (5-95% CI 1.23 to 1.76), $p = 1.9 \times 10^{-5}$. In contrast, no association with alleles of the same marker was found in AAD. Full genotype and allele frequencies for the rs1990670 IFIH1 marker are shown in table 1.

In contrast, there was no association found in either the GD or AAD patient cohorts when compared to healthy controls at the PD1.3 SNP (rs11568821). The minor allele frequency being 11.6% in the healthy control subjects, compared to 10.3% in the GD patients and 15.2% in the AAD cohort (full genotype and allele data are shown in table 2).

Heterogeneity testing according to the presence or absence of thyroid associated ophthalmopathy in the GD patients showed no significant difference in genotypes between the 2 groups for either marker (data not shown). Similarly, when comparing patients with isolated AAD to type 2 polyendocrinopathy, there were no significant differences in genotype frequencies at either locus (data not shown).

DISCUSSION

The first, exciting findings from a large-scale candidate variant study involving more than 6,500 non-synonymous single nucleotide polymorphisms in large cohorts of patients with type 1 diabetes revealed the *IFIH1* gene as a novel locus (9). In our current study, we confirm that the alanine carrying allele at *IFIH1* is also substantially associated with Graves' disease ($p=1.9 \times 10^{-5}$). This result is important for several reasons. Firstly, more than any previous form of genetic linkage or association analysis, genome-wide association studies test out numerous markers and there is a possibility of generating highly significant false-positive results on account of the multiple hypotheses implicit in such a study. In the work of Smyth and colleagues (9), the positive association at *IFIH1* was found in 1,924 subjects with T1D and replicated both in a second cohort of 2,329 T1D probands and in 2,134 trio families affected with T1D. While this initial analysis was robust by any standard, it is nevertheless still strongly reassuring to find that the association of autoimmune disease with *IFIH1* alleles has now been independently extended to include another autoimmune disorder, Graves' disease.

Secondly, IFIH1 is thought to have a role in protecting the host from viral infection by sensing viral nucleic acid in the cytoplasm and triggering a cellular anti-viral and apoptotic response (10,11). As Coxsackie and other enteroviral infections are epidemiologically linked to T1D incidence (17), it was suggested that IFIH1 polymorphism could form a molecular link between the specific viral trigger and the autoimmune response in T1D (9). The finding of strong association of IFIH1 alleles with GD, a condition with no established link to viral infection suggests that IFIH1 might have an endogenous immunoregulatory effect, unrelated to any specific role as a viral receptor.

Lastly, the association of IFIH1 alleles with GD in our population appears to be stronger than that with T1D, with an odds ratio for association of 1.47, in contrast to 1.16 found with T1D. While some of the variation in odds ratios may be stochastic, in particular relating to the slightly lower prevalence of the disease-associated alanine carrying IFIH1 allele in our local healthy control population compared to the UK-wide controls used in the diabetes study (57.0% vs 59.6%), there may be a genuinely stronger effect of IFIH1 in autoimmune thyroid diseases than in T1D, as has already been observed for alleles at the CTLA4 locus (18,19). Our analysis suggests that there is genuine heterogeneity in the strength of association at IFIH1 found in our GD cohort and in the T1D cohorts (an interaction term in a logistic regression was highly significant, $p=10^{-16}$). Conversely, our study does not have the power to exclude a small effect of IFIH1 in autoimmune Addison's disease pathogenesis. If the strength of effect in AAD is of the same order as that found in T1D (OR 1.16), more than 900 AAD probands and matched controls would be needed to show association. AAD is a relatively rare autoimmune endocrinopathy,

with a prevalence about 1 in 9000 in the UK (20) and sample cohorts of such a size currently do not exist. Collection and testing of such a larger Addison's patient cohort would be a valuable future goal.

In summary, this study confirms IFIH1 as a novel autoimmunity locus, extending its known role in type 1 diabetes to include a substantial role in Graves' disease susceptibility. We can find no evidence to support an effect at the PDCD1 locus on susceptibility to either Graves' or autoimmune Addison's disease, underlining the differences in pathogenesis between these organ-specific autoimmune disorders and the non-organ specific disorders, where PDCD1 polymorphism has been shown to have a role.

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Table 1. Genotype and allele frequencies for rs1990760 in Graves' disease, Addison's disease and healthy controls.

	Genotypes			Alleles		P value ^a		Allelic odds ratio (5-95% confidence intervals) ^a
	AA	AG	GG	A	G	<i>Genotypes</i>	<i>Alleles</i>	
Controls (n=446)	148 (33.2)	212 (47.5)	86 (19.3)	508 (57.0)	384 (43.0)			
Graves' disease (n=602)	266 (44.2)	264 (43.9)	72 (11.9)	796 (66.1)	408 (33.9)	1.4x10 ⁻⁴	1.9 x10 ⁻⁵	1.47 (1.23 to 1.76)
Addison's disease (n=204)	69 (33.8)	105 (51.5)	30 (14.7)	243 (59.6)	165 (40.4)	0.35	0.38	1.11 (0.88 to 1.41)

^a. P values and odds ratios are shown for the each of the patient cohorts in comparison to healthy controls

Table 2. Genotype and allele frequencies for rs11568821 in Graves' disease, Addison's disease and healthy controls.

	Genotypes (%)			Alleles (%)		P value ^a		Allelic odds ratio (5-95% confidence intervals) ^a
	AA	AG	GG	A	G	Genotypes	Alleles	
Controls (n=432)	6 (1.5)	88 (20.5)	338 (78)	100 (11.6)	764 (88.4)			
Graves' disease (n=596)	7 (1.2)	109 (18.3)	480 (80.5)	123 (10.3)	1069 (89.7)	0.66	0.37	1.13 (0.85 – 1.50)
Addison's disease (n=214)	4 (1.9)	57 (26.6)	153 (71.5)	65 (15.2);	363 (84.8)	0.17	0.07	0.73 (0.52 – 1.02)

^a P values and odds ratios are shown for the each of the patient cohorts in comparison to healthy controls