A haplotype of the CYP27B1 promoter is associated with autoimmune Addison’s disease but not with Graves’ disease in a UK population

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Abstract
Previous studies have suggested an association between alleles of the CYP27B1 (1-α hydroxylase) gene and autoimmune conditions. We have examined three single nucleotide polymorphisms (SNPs) that are located in the 5’ region and promoter of the CYP27B1 gene for association in a cohort of Graves’ disease and autoimmune Addison’s disease subjects from the UK. Genomic DNA samples from white patients with autoimmune Addison’s disease (n=104) and healthy controls (n=464) were genotyped by PCR-RFLP analysis for the SNPs at positions −1918, −1260 and −1077 5’ of the coding CYP27B1 sequences. The −1260 SNP was also examined in a cohort of patients with Graves’ disease (n=446). χ² testing of contingency tables was used to determine the significance of genotypes and alleles. Haplotype frequencies and linkage disequilibrium measures were estimated using the UNPHASED and HAPLOVIEW packages. Alleles at the three CYP27B1 SNPs were in tight linkage disequilibrium with each other and all showed association with autoimmune Addison’s disease. The maximum evidence for association was with the −1260 C allele (76.0% in Addison’s subjects versus 64.9% in controls; P=0.003; odds ratio 1.71 (9–95% confidence intervals, 1.20–2.44). A global test of significance for the common −1918 T, −1260 C and −1077 G haplotype was significant in Addison’s subjects compared with controls (P=0.01). In contrast, there was no association of alleles at the −1260 SNP with Graves’ disease. We are able to confirm that a CYP27B1 promoter allele is associated with autoimmune Addison’s disease, and extend this finding to include an associated promoter haplotype.

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Introduction
The pathogenesis of autoimmune endocrine disorders remains poorly defined, although genetic factors play a major role in the most common conditions (Brix et al. 2001, Vaidya et al. 2002, Tait & Gough 2003). In common with other complex traits, most autoimmune disorders are thought to have a multigenic basis; that is, in different individuals they are determined by a varying combination of susceptibility alleles in different genes. To date, alleles within the major histocompatibility complex (MHC; Pociot & McDermott 2002, Tait & Gough 2003), cytotoxic T lymphocyte antigen-4 (CTLA4) (Yanagawa et al. 1995, Heward et al. 1999, Vaidya et al. 1999, Ueda et al. 2003) and the lymphoid tyrosine phosphatase (LTP; Bottini et al. 2004, Smyth et al. 2004, Velaga et al. 2004) genes have been implicated in several autoimmune endocrinopathies including Type 1 diabetes, Graves’ disease (GD) and Addison’s disease. However, there is still a substantial component of the inherited susceptibility to these autoimmune conditions that remains unknown.

One method of identifying novel susceptibility genes for complex autoimmune disorders is to study polymorphisms in candidate genes that have been identified by functional investigations to have a role in the regulation of the immune response. The vitamin D-endocrine axis has a well-established influence on immune-system function (DeLuca & Cantorna 2001). Administration of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the biologically active form of vitamin D, has been shown to ameliorate murine autoimmunity in the nonobese diabetic (NOD) model (Mathieu et al. 1992) and in experimental autoimmune encephalomyelitis, a model of multiple sclerosis (Cantorna et al. 1996). In addition, there are epidemiological data linking vitamin D nutritional status in childhood with the prevalence of Type 1 diabetes (EURODIAB Substudy 2 Study Group 1999, Hypponen et al. 2001). 1-α Hydroxylase, encoded by the CYP27B1 gene, is the key enzyme in the production of 1,25(OH)₂D₃ from the inactive precursor 25-hydroxyvitamin D₃. A murine cyp27b1 knockout, in addition to the expected defects in calcium and bone homeostasis, displays disordered T lymphocyte subsets and cortical lymphadenopathy, suggesting autoimmunity (Panda et al. 2001).

Several investigators have suggested that vitamin D receptor (VDR) polymorphisms may have a role in
autoimmune conditions (Fukawaza et al. 1999, Ban et al. 2000, Pani et al. 2000, 2002), but comprehensive well-powered studies have failed to show significant association of VDR alleles with either Type 1 diabetes or GD (Collins et al. 2004, Nejentsev et al. 2004). In contrast, two recent small studies have shown association of CYP27B1 promoter alleles within trio families with Type 1 diabetes (Lopez et al. 2004a) and in a case-control design in cohorts of patients with Addison’s disease, GD, Hashimoto’s thyroiditis and Type 1 diabetes from the German population (Lopez et al. 2004b). Replication of these findings in an independent patient cohort would be an important step forward in confirming these potentially exciting findings. In this report, we have studied three single nucleotide polymorphisms (SNPs) upstream of the CYP27B1 coding region in UK patient cohorts with autoimmune Addison’s disease (AAD) and GD.

Subjects and methods

Subjects

104 AAD probands were recruited from endocrine clinics from the Newcastle upon Tyne Hospitals Trust and surrounding district hospitals. The clinical characteristics of this AAD cohort have been reported previously (Vaidya et al. 2000). GD subjects (n=446) were also recruited from local endocrine clinics and clinical details of this cohort have been published previously (Houston et al. 2004). Healthy controls with no clinical features or family history of autoimmune disease were recruited from offices and factories in Newcastle. All case and control patients were of white ethnicity and all had parents born in the north-east of England. The studies were approved by the Newcastle and North Tyneside ethics committee.

Genotyping

Genomic DNA was isolated from peripheral blood samples using the Nucleon BACC2 kit. Three SNPs in the CYP27B1 gene were examined, two of which (−1260 and −1077) were in the promoter region, the −1918 SNP being in the region immediately 5’ to the promoter. AAD patients (n=104) and controls (n=464) were genotyped for all three SNPs. GD probands (n=446) were also genotyped for the most associated promoter SNP (−1260). Genomic DNA was amplified by PCR and genotyping was carried out by RFLP. The −1260 and −1077 SNPs were amplified in a single product using the primers F, 5’-GACAAGGTGAAGAGCCAGAAGCCAG-3’, and R, 5’-CTGACCTGGTCTAGCTGTCGAGGC-3’, and an annealing temperature of 62°C. The enzymes Tsp509I, TfiI and TaqI were used for the −1918, −1260 and −1077 SNPs respectively. The −1918 assay used a forced restriction site. PCR products were digested overnight at 65°C and electrophoresed on agarose gels. Genotypes were confirmed by direct DNA sequencing in individuals of each different genotype.

Statistical analysis

The case-control association studies were analysed by χ² testing using 2×3 and 2×2 contingency tables of genotype and allele frequencies, respectively. Haplotype frequencies were estimated using the UNPHASED package (Dudbridge 2003) and linkage disequilibrium (D’) measures were calculated using HAPLOVIEW (Barrett et al. 2004). Odds ratios and confidence intervals (CIs) were calculated using Woolf’s method (Woolf 1995). A power calculation, based on the observed allele frequencies of the −1260 SNP found in the previous study (Lopez et al. 2004b), showed that our GD cohort had more than 90% power to detect an effect with an odds ratio of 1.5 (a=0.05).

Results

Genotyping of all three CYP27B1 SNPs was performed in 104 Addison’s disease patients and 464 healthy controls. The GD cohort (n=446) was also genotyped for the most associated SNP (−1260). All genotype frequencies were in Hardy–Weinberg equilibrium. Table 1 shows full genotype- and allele-frequency data. All three of the CYP27B1 SNPs were associated with AAD, with P values for allele frequencies ranging between 0.02 and 0.003 compared with healthy controls. The C allele at the −1260 promoter SNP was the allele most associated with AAD, showing an odds ratio of 1.71 (5–95% CI, 1.20–2.44). In contrast, there was no association in our large cohort of GD subjects at this marker (Table 1), despite good statistical power (>90%) to detect a similar effect in size to that observed in the AAD cohort.

Analysis of haplotypes showed that all three SNPs were in tight linkage disequilibrium with each other, with pairwise D’ values as follows: [−1918] = −0.95 [−1260] = 0.98 [−1077]. A disease-associated haplotype could therefore be established containing the alleles −1918 T, −1260 C and −1077 G. This haplotype was found in 75.8% of AAD subjects as compared with only 66.7% of control individuals (P=0.01; odds ratio, 1.56; 5–95% CI, 1.20–2.44).

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Discussion

Our study confirms the previous findings of association of CYP27B1 polymorphisms with AAD (Lopez et al. 2004b). We are able to extend these findings by demonstrating an associated three-marker haplotype of the CYP27B1 promoter that appears to confer susceptibility to AAD. In contrast, we did not find evidence for association at this locus in our substantial cohort of GD subjects from the UK population, despite good power to detect a moderate effect.

1,25(OH)2-D3 is known to have important effects on several cellular compartments of the immune system, including T lymphocyte subsets, dendritic cells and macrophages. However, B lymphocytes, in contrast to most other immune-system cell types, have very low levels of VDR expression, suggesting that the immunomodulatory influence of 1,25(OH)2-D3 may be more marked in cell-mediated forms of immune attack, such as those involved in Th1-type immune responses (DeLuca & Cantorna 2001). Thus, it is possible that there are differences in the influences of CYP27B1 polymorphism between the classically cell-mediated forms of autoimmune disease such as Type 1 diabetes and AAD, and the conditions where a humoral immune response is thought to predominate, such as GD.

Of interest, the gene encoding the VDR has been subject to intense study in a variety of conditions including some autoimmune disorders. Whereas, based on evidence from large studies, it seems likely that common VDR polymorphisms do not play a substantial role in the pathogenesis of either GD or Type 1 diabetes (Collins et al. 2004, Nejentsev et al. 2004), some of the modest allelic associations found within VDR could be consistent with a real susceptibility gene in the same chromosomal region. CYP27B1 and VDR both map to the long arm of chromosome 12; however, the 10·1 Mbp separating them is almost certainly too large a distance for the associations found in one gene to be explained by linkage disequilibrium with the other.

The disease-associated CYP27B1 promoter haplotype that we have identified in this study may lead to significant functional variation in CYP27B1 transcription and hence to differences in 1α-hydroxylase activity. Thus, these genetic changes may be responsible for determining the differential effect of the alleles on immune system function. In favour of this suggestion, the most associated SNP allele, −1260 C, changes a consensus CDX2 transcription factor-binding site (TATTT to TCTTT) within the CYP27B1 promoter. Thus the −1260 promoter SNP has the potential to directly mediate the functional effect of the genetic variation on this common haplotype. CDX2 is a caudal type homeobox transcription factor that plays an important role in the intestine-specific transcription of the VDR gene (Yamamoto et al. 1999). A SNP in the CDX2 site of the VDR gene promoter is known to affect transcription of the VDR gene and has shown to be associated with differences in bone mineral density and fracture risk (Fang et al. 2003). However, CDX2 is not

### Table 1 Genotype frequencies for Graves’ and Addison’s disease patients at CYP27B1 SNPs

<table>
<thead>
<tr>
<th>Genotype/allele</th>
<th>GD (n=446)</th>
<th>AAD (n=104)</th>
<th>Controls (n=464)</th>
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<td>G</td>
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</table>

Genotype P values (2 df): GD versus controls, *−1260, P=0·55 (not significant); AAD versus controls, ‡−1918, P=0·045; ¶−1260, P=0·01; ††−1077, P=0·045.
Allele P values (1 df): GD versus controls, †−1260, P=0·524 (not significant); AAD versus controls, §−1918, P=0·0097; **−1260, P=0·0031 (odds ratio, 1·71; 5–9% CI, 1·20–2·44); ††−1077, P=0·0181.
currently recognized as an immune system transcription factor, and a comprehensive analysis of all the genetic variants in the CYP27B1 gene in larger patient cohorts is required to fully elucidate the extent of disease-associated linkage disequilibrium, and determine the candidacy of other polymorphisms in this region.

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