Polymorphisms of the CYP2D6 gene increase susceptibility to ankylosing spondylitis

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Ankylosing spondylitis (AS) is a common and highly familial rheumatic disorder. The sibling recurrence risk ratio for the disease is 63 and heritability assessed in twins >90%. Although MHC genes, including HLA-B27, contribute only 20–50% of the genetic risk for the disease, no non-MHC gene has yet been convincingly demonstrated to influence either susceptibility to the disease or its phenotypic expression. Previous linkage and association studies have suggested the presence of a susceptibility gene for AS close to, or within, the cytochrome P450 2D6 gene (CYP2D6, debrisoquine hydroxylase) located at chromosome 22q13.1. We performed a linkage study of chromosome 22 in 200 families with AS affected sibling-pairs. Association of alleles of the CYP2D6 gene was examined by both case–control and within-family means. For case–control studies, 617 unrelated individuals with AS (361 probands from sibling-pair and parent–case trio families and 256 unrelated non-familial sporadic cases) and 402 healthy ethnically matched controls were employed. For within-family association studies, 361 families including 161 parent–case trios and 200 affected sibling-pair families were employed. Homozygosity for poor metabolizer alleles was found to be associated with AS. Heterozygosity for the most frequent poor metabolizer allele (CYP2D6*4) was not associated with increased susceptibility to AS. Significant within-family association of CYP2D6*4 alleles and AS was demonstrated. Weak linkage was also demonstrated between CYP2D6 and AS. We postulate that altered metabolism of a natural toxin or antigen by the CYP2D6 gene may increase susceptibility to AS.
### RESULTS

The CYP2D6 allele frequencies in each patient collection and controls are given in Table 1. No significant difference in allele frequencies was observed between our controls and those previously reported by Beyeler et al. (Table 1) (11). Therefore, results are reported both separately and pooled together with this previously reported study. Neither case nor control genotype frequencies vary significantly from Hardy–Weinberg equilibrium.

Examining cases and controls only from the current study, significant association was observed between homozygosity for CYP2D6*4 and AS [genotype relative risk (GRR) = 2.1, 95% confidence interval 1.3–3.4, \( P = 0.002 \)]. In contrast, the risk of disease among heterozygotes was not increased, the overall GRR being 1.1 (95% confidence interval 0.8–1.4, \( P = 0.5 \)). Pooling all patients and controls, homozygosity for the CYP2D6*4 allele was strongly associated with AS, with a GRR of 2.1 (95% confidence interval 1.5–3.1, \( P = 0.0005 \)). Again, heterozygosity for allele 4 was not associated with AS (GRR = 1.0, 95% confidence interval 0.9–1.2, \( P = 0.7 \)).

CYP2D6*4 homozygosity and CYP2D6*4/CYP2D6*5 heterozygosity both result in the poor metabolizer phenotype. Considering both these poor metabolizer genotypes, the association with AS is highly significant both in the current study (GRR = 2.2, 95% confidence interval 1.4–3.6, \( P = 0.0007 \)) and with the pooled results (GRR = 2.1, 95% confidence interval 1.5–3.1, \( P = 0.0003 \)). Significant transmisison disequilibrium of the CYP2D6 polymorphism was observed using Transmit \( [\chi^2 = 7.3 \text{ (1 d.f.)}, \text{empirical } P = 0.014] \).

Weak linkage of the CYP2D6 polymorphism and AS was observed with a LOD score of 0.9 \( [P = 0.02, \text{mean identical-by-descent (IBD) sharing 0.6}] \). Weak linkage was also observed for the microsatellite maker CYP2D, with a LOD score of 0.6 \( (P = 0.05) \). For the CYP2D6 polymorphism the proportion of families sharing one allele was considerably reduced from the expected \( (z_1 \text{ expected 0.5, observed 0.4}) \), implying the presence of dominance variance. Multipoint LOD scores at the CYP2D6 locus were slightly lower than single-point scores (LOD = 0.5). No other marker showed significant evidence of linkage (mean IBD sharing < 0.55, \( P > 0.05 \)).

### DISCUSSION

This report confirms the previously reported finding of significant association between poor metabolizer genotypes and AS (11). Within-family association was also demonstrated for the CYP2D6*4 polymorphism, demonstrating that the case–control findings indicate true association rather than the presence of population stratification. Weak evidence of linkage was also noted both for the CYP2D intragenic microsatellite and the CYP2D6 gene.

CYP2D6 poor metabolizer phenotype can be due to at least 15 different genetic variants of the CYP2D6 gene (13). Homozygosity for CYP2D6*4 is genetically responsible for \( \sim 75\% \) of poor metabolizers (14,15). The lack of association between heterozygosity for CYP2D6*4 and AS suggests either that we have observed true association with a recessively acting polymorphism, or that CYP2D6*4 is in strong linkage disequilibrium with another recessively acting allele. IBD sharing analysis at the CYP2D6 locus revealed a marked reduction in the sharing of one allele IBD \( (z_1) \), supporting the presence of dominance variance at this locus. Where a gene acts codominantly, the proportion \( z_1 \) is 0.5 and deviation below this occurs with dominant or recessive acting genes. In the current study the reduction of \( z_1 \) from the expected value of 0.5 to the observed value 0.4 is consistent with a recessive genetic effect.

The use of the GRR statistic depends on the assumption that the control genotypes are in Hardy–Weinberg equilibrium. In this circumstance the statistic is more powerful than the standard chi-squared statistic (16). In this study the control genotypes do not deviate significantly from those expected under Hardy–Weinberg equilibrium, and therefore the use of the GRR is justified.

While case–control studies are susceptible to error due to population stratification, the Transmit transmission disequilibrium statistic has been shown to be highly robust to violation of the assumption of population stratification (17). The finding of significant association using this statistic is therefore highly significant in that it indicates that the case–control results are unlikely to be due to this source of error. The CYP2D6 allele frequencies we observed are very similar to those reported in other studies of British Caucasians both from London (18) and the northeast of England (11), also supporting the conclusion that true association is the cause of the findings reported here.

### Table 1. CYP2D6 genotype frequencies in patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Current study</th>
<th>Historic study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients N (%)</td>
<td>Controls N (%)</td>
</tr>
<tr>
<td>Non-CYP2D6*4 homozygotes</td>
<td>383 (62)</td>
<td>267 (66)</td>
</tr>
<tr>
<td>CYP2D6*4/wild-type heterozygotes</td>
<td>188 (30)</td>
<td>118 (29)</td>
</tr>
<tr>
<td>CYP2D6*4 homozygotes</td>
<td>43 (7)</td>
<td>16 (4)</td>
</tr>
<tr>
<td>CYP2D6*4/5</td>
<td>3 (0)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>617</td>
<td>402</td>
</tr>
</tbody>
</table>

Historic patients and controls refer to those reported by Beyeler et al. (11). Wild-type refers to non-CYP2D6*4 and non-CYP2D6*5 alleles.
Poor metabolizer phenotype has been associated with a wide range of diseases including Parkinson’s disease (19), cancers (20), scleroderma (21) and systemic lupus erythematosus (22), although replication of the latter two findings has not been reported. Whilst CYP2D6 is known to be involved in metabolism of a wide range of drugs, no endogenous CYP2D6 substrate has yet been identified, and therefore its biological function remains uncertain. It has been postulated that poor metabolism of xenobiotics (i.e. drugs, metals, industrial and naturally occurring chemicals) or plant toxins by the defective CYP2D6 variants may explain these associations (22). Xenobiotics are known to have numerous pro-inflammatory effects on T-cells (23), which may be critical cells in the pathophysiology of B27-related arthritis (24). These effects include uncovering of cryptic peptides by hapten formation producing autoimmune T-cell reactions, and direct recognition of xenobiotics or their metabolites by T-cells (23). Well-known examples of autoimmune diseases due to xenobiotics include Stevens–Johnson syndrome and dihydralazine-induced systemic lupus erythematosus, in which impaired drug metabolism is a significant disease risk factor. Whilst these diseases are clearly quite different to AS, they illustrate that impaired xenobiotic metabolism can induce autoimmune diseases in humans.

This study strongly suggests that dysfunction of the CYP2D6 gene increases the risk of AS, although only contributing a small proportion of the overall risk of the disease. Further studies of enzymes involved in xenobiotic metabolism are warranted in AS, and in other T-cell mediated autoimmune diseases.

MATERIALS AND METHODS

Patients

We studied linkage of the CYP2D6 gene, and association of the main poor metabolizer genotype in 617 unrelated AS patients and 402 healthy controls, and in 361 families with AS consisting of 161 parent–case trios and 200 affected sibling-pair families, containing 276 affected sibling-pairs. The 105 affected sibling-pair families used in our initial whole genome screen are included in the affected sibling-pair families used in this study. In the parent–case trios all parents were available for genotyping, whereas of the affected sibling-pair families, 71 had both parents available, 63 one parent and 66 neither parent. The probands from these families were also used in the case–control association studies. AS was defined by the diagnostic criteria of the modified New York criteria for AS consisting of radiographic and clinical criteria (25). Four hundred and two control healthy blood donors were recruited through the Oxford Regional Transfusion Service. An additional panel of 662 UK historic controls and 54 AS patients previously reported were used in some of the analyses (11). All patients and controls were British Caucasian subjects. Ethics approval for this study was obtained from the Central Oxford Research Ethics Committee.

Laboratory methods

We used a modified version of the typing method reported by Beyeler et al. (11), by which all samples were genotyped for the CYP2D6*4 allele. This method involves a restriction digest using the enzyme BstNI, which cuts at position 1934 in wild-type but not mutant alleles. Because this method fails to distinguish between reactions where the restriction digest has failed and homozygous mutant samples, we altered the position of the 5′-PCR primer to also include a non-polymorphic BstNI restriction site at position 1772. The primers used were D6L2 (5′-GGT GTT CCT CGC GCG CTA TG-3′) and P5 (5′-CTC GGT CTC TCG CTC CGC AC-3′) which produce a 421 bp product. The inclusion of this second restriction site produces a constant 77 bp fragment, so that failure of digestion can clearly be distinguished from homozygous mutant samples. The primer D6L2 has a deliberate mismatch of the 10th base to distinguish between reactions where the restriction digest has failed for genotyping, whereas of the affected sibling-pair families, < 0.1% (11) and were therefore not specifically genotyped in this study. We typed all apparent CYP2D6*4 homozygotes for CYP2D6*5 using a variation of a previously reported method (26). The same PCR primers were used, but rather than ‘long-range’ DNA polymerase, we employed standard DNA polymerase (Bioline) and Q-solution (Qiagen, Crawley, UK), which gave more reliable amplification of this GC-rich region.

In addition to the marker CYP2D, 10 chromosome 22 fluorescently tagged microsatellites spread across chromosome 22 (D22S1158, D22S1173, D22S1176, D22S274, D22S277, D22S280, D22S283, D22S315, D22S420, D22S423—average marker spacing 4.6 cM) were PCR amplified and products separated on ABI 373 semiautomated sequencers by standard means (3). Mendelian inheritance was verified both manually and using the program Pedcheck (27).

Statistical methods

Non-parametric two-point linkage analysis was performed using the package, Analyze (28). IBD sharing assessment and
multipoint analysis was performed using the non-parametric program MapMaker/Sibs, conservatively weighting the numbers of affected sibling-pairs per family by 2/number of affecteds (29). GRRs were calculated according to the method of Lathrop (16). In this method, genotype frequencies and their variances are calculated from the observed allele frequencies assuming Hardy–Weinberg equilibrium, resulting in a power gain because the statistic variances are smaller compared with those from standard contingency table methods. The method is valid to use in our case, as the control genotype frequencies are in Hardy–Weinberg equilibrium. Unlike the GRR definition of Risch and Merikangas (30), this method makes no assumptions about the relative GRR of homozygotes and heterozygotes. Within-family association was studied using the program Transmit (17), a form of transmission–disequilibrium test, which permits use of families where parental genotypes may be missing. P-values were obtained by bootstrap simulation using 10,000 replicates. When using bootstrapping, the statistic is robust to inclusion of multiple affected offspring in each family, even in the presence of linkage.

REFERENCES


