Cis- and trans- loci influence expression of the schizophrenia susceptibility gene \textit{DTNBP1}

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Susceptibility to complex disease appears to be partly mediated by heritable differences in gene expression. Where \textit{cis}-acting effects on a gene’s expression influence disease susceptibility, other genes containing polymorphism with a \textit{trans}-acting effect on expression of that gene may also be expected to modulate risk. Use of the expression of an identified disease gene as an endophenotype for quantitative linkage analysis may therefore provide a powerful method for mapping loci that modulate disease susceptibility. We performed genome-wide linkage analysis on expression of \textit{dystrobrevin binding protein 1} (\textit{DTNBP1}), a well-supported susceptibility gene for schizophrenia, in large CEPH pedigrees. We observed genome-wide significant evidence for linkage at the \textit{DTNBP1} locus on chromosome 6p22, and demonstrated that this reflects variable \textit{cis}-acting effects on \textit{DTNBP1} expression. In addition, we observed genome-wide suggestive evidence for linkage of \textit{DTNBP1} expression to chromosome 8p, suggesting a locus that exerts a \textit{trans}-acting effect on \textit{DTNBP1} expression. The region of linkage to \textit{DTNBP1} expression on chromosome 8 is contiguous with linkage findings based upon the clinical schizophrenia phenotype, and contains another well-supported schizophrenia susceptibility gene, \textit{neuregulin-1} (\textit{NRG1}). Our data provide complementary evidence for chromosome 8p as a susceptibility locus for schizophrenia, and suggest that genetic variation within this region may influence risk, at least in part, through effects on \textit{DTNBP1} expression.

\textbf{INTRODUCTION}

Heritable differences in gene expression are considered to play an important role in complex traits, including susceptibility to human disease (1,2). This hypothesis is supported directly through tests of association with regulatory polymorphism (3,4), and indirectly through the cloning of susceptibility genes in which coding sequence variants do not fully account for disease association (5–7).

Genetic influences on gene expression may be either \textit{cis}- or \textit{trans}- acting. \textit{Cis}-acting polymorphisms are located within the same functional unit as the influenced gene, although they may be some distance from the coding sequence (8). \textit{Trans}-acting polymorphisms are not physically linked to the \textit{trans}-regulated gene; rather, their effects are exerted through functional (\textit{cis}-acting) consequences on distinct genes, which in turn influence expression of the target gene.

It is possible to map polymorphic regulatory loci by using gene expression as a quantitative trait for linkage analysis (9–13). Large-scale studies in humans have indicated that a significant proportion of the heritable variance in gene expression is attributable to \textit{trans}-acting polymorphism (11,12). For the growing number of reported instances in which variation at a disease locus is found to promote susceptibility through \textit{cis}-acting effects on gene expression, e.g. (3,4,14), this observation has potentially important implications: if disease susceptibility is influenced by variable \textit{cis}-acting effects on a gene’s expression, then other genes

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containing polymorphism with a trans-acting effect on expression of the disease gene may also be expected to modulate disease susceptibility. For complex diseases, these loci are likely to confer a greater effect on the intermediate expression phenotype than on the clinical phenotype, and thus should be more easily detected. Use of a disease gene’s expression as an endophenotype for quantitative linkage analysis may therefore provide a powerful method for mapping loci that modulate disease susceptibility.

The dystrobrevin binding protein 1 gene (DTNBP1), encoding dysbindin, is one of the best-supported susceptibility genes for schizophrenia (15,16). Whereas a rare coding mutation in DTNBP1 has been found to cause a form of Hermansky–Pudlak syndrome (17), the absence of schizophrenia-associated polymorphisms in DTNBP1 protein coding sequence strongly suggests that susceptibility is conferred by cis-acting variants that influence DTNBP1 expression. Such variants have been shown to exist in DTNBP1, and to operate in human brain (18). Moreover, there is direct evidence linking schizophrenia risk haplotypes at the DTNBP1 locus with reduced allelic expression of DTNBP1 (19).

Two independent post-mortem studies have found reduced DTNBP1 expression in the brains of schizophrenia cases compared with controls (20,21). Importantly, the schizophrenia cases used in these studies were not genetically selected for risk haplotypes at the DTNBP1 locus, and therefore the observed differences in expression between cases and controls are unlikely to reflect cis-acting genetic factors alone. Rather, the reduction in DTNBP1 expression in schizophrenia is likely to result in part from trans-acting risk factors, which may be both genetic and environmental.

The aim of the present study was to map genetic loci that affect DTNBP1 expression, under the assumption that such loci are a priori also likely to influence susceptibility to schizophrenia. We performed genome-wide quantitative linkage analysis on real-time PCR measures of DTNBP1 expression using lymphoblastoid RNA from CEPH families. Advantages of this resource for linkage analysis are that large sibships are available that have already been genotyped for a genome-wide SNP map (22). We obtained evidence not only for the cis-acting locus with reduced allelic expression at 8p (Fig. 3), suggesting the presence of a polymorphic locus affecting DTNBP1 expression (19), but also for a polymorphic locus affecting DTNBP1 expression in trans.

RESULTS

Real-time quantitative PCR measures of lymphoblastoid DTNBP1 expression were obtained for 200 individuals, representing 26 large CEPH sibships. These measures were used for genome-wide quantitative linkage analysis, in order to map loci that influence DTNBP1 expression. Strongest evidence for linkage was observed at the DTNBP1 locus on chromosome 6p22, consistent with cis-acting influences on DTNBP1 expression (Fig. 1). A maximum LOD of 3.2 was observed at 31.4 cM, between markers rs2038016 (30.2 cM, 14.8 Mb) and rs651394 (37.3 cM, 16.9 Mb), that flank the DTNBP1 locus at 15.6–15.8 Mb. Simulation tests showed this linkage to be genome-wide significant (0.03 peaks/genome scan).

![Figure 1. Linkage to DTNBP1 expression on human chromosome 6. Distance from p-ter (X-axis) is given in sex-averaged cM according to the SNP linkage map constructed by Matise et al. (22). A maximum LOD of 3.2 was observed at 31.4 cM, between markers rs2038016 (30.2 cM, 14.8 Mb) and rs651394 (37.3 cM, 16.9 Mb) that flank the DTNBP1 locus at 15.6–15.8 Mb.](https://academic.oup.com/hmg/article-abstract/17/8/1169/651921)

To verify that the linkage detected on chromosome 6p reflects polymorphic cis-acting effects on DTNBP1 expression, we assayed DTNBP1 in the lymphoblastoid cDNA samples using measures of relative allelic expression. This method effectively controls for trans-acting influences by measuring the relative expression of each gene copy within individual samples. Departure from the 1:1 ratio of the two alleles observed in genomic DNA ratio in any cDNA sample is indicative of heterozygosity for a cis-acting effect on expression. A total of 56 individuals were heterozygous for the exonic DTNBP1 ‘tag’ A>G polymorphism rs1047631, and therefore informative for relative allelic expression analysis. As with our previous findings in brain (19), a wide range of individual cDNA ratios was observed (Fig. 2), with 11 of the assayed samples showing differences in allelic expression of at least 50% (allelic ratios of <0.67 or >1.5), thus confirming variable cis-acting influences on DTNBP1 expression operating in the lymphoblastoid cells.

A second, broader linkage peak was observed on chromosome 8p (Fig. 3), suggesting the presence of a polymorphic locus (or loci) affecting DTNBP1 expression in trans. The maximum LOD of 2.77 was observed at 50.17 cM (chromosome 8p12), between the markers rs2169315 (46.5 cM, 28.1 Mb) and rs726908 (52 cM, 32.2 Mb). Simulation analysis showed this linkage to be genome-wide suggestive (0.16 peaks/genome scan). The LOD-1 interval for this peak extended from 44.2 cM (near rs1351499 at 26.9 Mb) to 56 cM (near rs1481065 at 40.0 Mb). A smaller, more telomeric peak observed at 26.8 cM (chromosome 8p22) yielded a LOD of 1.82 that did not reach statistical significance. Maximum LOD scores on all other chromosomes did not exceed 1.5.

DISCUSSION

As a novel approach to seek susceptibility loci for complex diseases, we have performed quantitative linkage analysis on the expression of DTNBP1, a well-supported susceptibility
gene for schizophrenia (16). We observed genome-wide significant evidence for linkage at the DTNB1 locus, consistent with cis-acting polymorphic influences on DTNB1 expression. Moreover, we also obtained evidence for linkage between DTNB1 expression and markers on chromosome 8p, suggesting a locus that exerts a trans-acting effect on DTNB1 expression, and which, through this mechanism, may also promote schizophrenia susceptibility.

Although human brain would (presumably) be the tissue of choice for studying regulatory influences on a schizophrenia susceptibility gene, this is not presently feasible for the type of study we have undertaken. Instead, we elected to study DTNB1 expression in lymphoblastoid cell lines from the CEPH pedigrees. The advantages of this resource for the present study are that: (i) linkage studies of gene expression require tissue from related individuals, a resource that is not readily acquired with respect to brain, (ii) the assayed samples have previously been genotyped across the whole genome with SNPs at a density suitable for linkage and (iii) cultured cell lines may be expected to minimize potential confounding influences on gene expression arising from tissue preparation, environmental variation and cellular heterogeneity.

The observed linkage to the DTNB1 locus is consistent with our previous finding, using different methodology, of polymorphic cis-acting influences on DTNB1 expression (19). That the linkage signal on chromosome 6p indeed reflects cis-acting regulatory variation was confirmed by analysis of DTNB1 relative allelic expression in the same lymphoblastoid RNA samples. This demonstrates the power of our study to detect genuine eQTL, and also the validity of our measures of the DTNB1 expression phenotype. It therefore suggests that the observed linkage of this phenotype to an additional, trans-acting, locus on chromosome 8p is also valid, although at a level of significance just short of genomewide significance.

This study is, to our knowledge, the first to provide evidence of a polymorphic locus affecting expression of a known disease susceptibility gene in trans. It should be noted that the linkage peak is broad and verification of the putative trans-acting locus on chromosome 8p will of course require identification of the causal gene(s). It is, however, noteworthy that this locus is, along with chromosome 6p24–p22, one of the most reproducible linkage regions for schizophrenia (16,23,24). Our data provide complementary evidence for chromosome 8p as a susceptibility locus for schizophrenia, and suggest that genetic variation within this region may influence risk, at least in part, through effects on DTNB1 expression.

A number of genes on chromosome 8p have been reported to show evidence for genetic association with schizophrenia. For none is the evidence implicating the gene in schizophrenia definitive, but we nevertheless list those genes within our broad linkage region where there is more than one positive report of association (Table 1). It is of interest that our maximum linkage on chromosome 8 was observed close to NRGI, located at 31.6–32.7 Mb, which, along with DTNB1, is probably the best-supported gene for schizophrenia anywhere in the genome (25,26). As with DTNB1, there have been reports of altered NRGI expression in schizophrenia (27,28). Intriguingly, there is also evidence that NRGI can regulate gene expression by binding to the zinc finger protein Eos (29). However, it is important to stress that there is as yet no evidence for regulation of DTNB1 by any NRGI isoform. The region of linkage to DTNB1 expression on chromosome 8p, in common with the region of linkage to the schizophrenia phenotype itself, is broad and encompasses many genes. Rather than conclude in favour of any particular candidate, we would urge a systematic approach in examining the region. It has proven possible to narrow candidate regulatory regions for gene expression phenotypes through genetic association using dense marker sets (30).

Use of DTNB1 expression as a phenotype for genetic association analysis may therefore help determine the variant(s) in this region conferring susceptibility to schizophrenia.

In summary, we present a novel approach for identifying susceptibility loci for complex disease, based on the use of a disease gene’s expression as an endophenotype for quantitative linkage analysis. The results of the present study suggest...
that expression of DTNBPI is influenced not only by cis-acting variation at the DTNBPI locus, which appears to mediate association between this gene and schizophrenia (19), but also by trans-acting polymorphism on chromosome 8p. These data provide complementary evidence for chromosome 8p as a schizophrenia susceptibility locus, and suggest that variants within the region modulate risk, at least in part, through downstream effects on DTNBPI expression.

**MATERIALS AND METHODS**

**Samples**

Utah CEPH pedigrees (1331, 1332, 1362, 1416, 1413, 1347, 1333, 1341, 1340, 1345, 1344, 1346, 1349, 1418, 1423, 13291, 13293, 1421, 1334, 1408, 1424, 1454, 1354, 1356, 1333, 1341, 1340, 1345, 1344, 1346, 1349, 1418, 1423, 13291, 13293, 1421, 1334, 1408, 1424, 1454, 1354, 1356, 1333, 1341, 1340, 1345, 1344, 1346, 1349, 1418, 1423, 13292, 1420) were selected on the basis that they had a minimum sibship size of 6 and had been genotyped with adequate coverage for SNP linkage (22). No phenotypic information is available regarding the samples. Lymphoblastoid cell lines were obtained from Coriell Cell Repositories and grown in RPMI 1640, supplemented with 15% fetal calf serum, 1% penicillin and streptomycin mix and 1% glutamine (Invitrogen). Cells were harvested at a density of 0.6–1×10^6 cells/ml by centrifuging for 10 min at 375 g. Cell pellets were washed in PBS, centrifuged for 4 min at 1000 g and stored at −80°C. Total RNA was extracted from cell pellets using the RNeasy Kit (Ambion) and DNase treated. RNA did not yield detectable levels of PCR product in the absence of reverse transcription. RNA was quantified using a spectrophotometer, and those samples showing low yields (<200 ng/μl) or 260/280 nm ratio <1.8 were excluded. RNA from the remaining 200 individuals was reverse transcribed in a total reaction volume of 20 μl, containing ~1 μg total RNA, random decamers and reagents within the RETROscript kit (Ambion) following the manufacturer’s protocol. RNA from each individual was reverse-transcribed on three separate occasions. cDNA samples were diluted 1/10 prior to real-time PCR.

**Gene expression measures**

Gene expression was measured by real-time quantitative PCR using TaqMan Gene Expression Assays (Applied Biosystems). 8p22-12 region for which there is more than one positive report of genetic association with schizophrenia, as cited in the SchizophreniaGene Database

**Table 1. Genes within the chromosome 8p22-12 region for which there is more than one positive report of genetic association with schizophrenia, as cited in the SchizophreniaGene Database**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position, Mb</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC18A1</td>
<td>20.0–20.1</td>
</tr>
<tr>
<td>PPP3CC</td>
<td>22.3–22.5</td>
</tr>
<tr>
<td>DPTSL2</td>
<td>26.4–26.6</td>
</tr>
<tr>
<td>FZD3</td>
<td>28.4–28.5</td>
</tr>
<tr>
<td>NRG1</td>
<td>31.6–32.7</td>
</tr>
</tbody>
</table>

Gene expression was measured by real-time quantitative PCR using TaqMan assay Hs01105864_m1, which contains primers and a probe that span exons 4 and 5 of the major DTNBPI transcript variants 1 (GI: 34304371) and 2 (GI: 34304367). In order to control for inter-sample differences in mRNA input or efficiency of reverse transcription, DTNBPI measures were normalized by concurrent measures of the RPS23 gene, using Taqman assay Hs01374150_g1. RPS23 was selected on the basis of an analysis of the microarray data generated by Morley et al. (12) (NCBI Gene Expression Omnibus accession number GSE1485), which showed high expression and low inter-subject variability of this gene in lymphoblastoid RNA. In addition, pilot measures of several unrelated target genes showed greater correlation with measures of RPS23 than with those of either 18S or ACTB in our lymphoblastoid samples. To ensure that real-time PCR measures were not influenced by polymorphism within the primer or probe target sequence, all exons included in TaqMan assays were screened for mutations in the parents of each sib-ship using a LightScanner mutation detection system (Idaho Technology Inc.), a method we have shown detects sequence variation with very high sensitivity (N.M.W. and S. Dwyer, unpublished data).

Real-time PCR reactions were performed in a total volume of 20 μl, containing 9 μl cDNA, 10 μl TaqMan Universal PCR mix (Applied Biosystems), primers at a concentration of 900 nM and probes at 250 nM. Three separate assays were performed, using independent cDNA preparations for each. For individual cDNA samples within each assay, reactions were performed for both DTNBPI and RPS23 in triplicate. Reactions were quantitated against a standard curve constructed by serial dilution of pooled lymphoblast cDNA, which was used at the same concentrations for each assayed 96-well plate. PCR was carried out on an ABI 7000 Sequence Detection System (Applied Biosystems), with an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Raw cycle threshold (C_T) values were obtained using SDS 2.0 software (Applied Biosystems). Standard curve dilutions were given arbitrary values based on their relative concentration, and the log of these values plotted against the measured C_T values. The slope (s) and y-axis intercept (i) of the trendline were used to calculate the relative log expression (log e) values for each of the individual unknown reactions based on their C_T values and the equation log e = (C_T − i)/s. The log value was then transformed to give an expression value for each reaction. For each assay, a mean value for both DTNBPI and RPS23 was obtained for each individual based on the expression values derived from the three replicate reactions. Quantitative linkage analysis was performed on the mean of the standardized residual values derived from regressing mean DTNBPI measures against mean RPS23 measures for each of the three independent assays. This was done to minimize the effect of random variation between assays on the linkage analysis. A histogram of these data is provided as supplementary data.
of 3.9 cM (22). SNP genotypes for all assayed individuals were downloaded from The SNP Consortium database. Distances from p-ter are given in sex-averaged cM according to Matise et al. (22). Aliases for The SNP Consortium database SNPs were obtained from NCBI dbSNP. Physical positions of linkage regions are given according to the UCSC Genome Bioinformatics March 2006 human genome assembly (http://genome.ucsc.edu/cgi-bin/hgGateway). Analyses were restricted to the autosomal chromosomes due to the poor coverage of chromosome X by the SNP Consortium data.

Quantitative trait linkage analysis

Multipoint quantitative trait linkage analysis was carried out on the mean residuals (as described earlier) using the variance component method implemented in MERLIN (31). The variance component method is one of the most powerful methods for quantitative trait linkage analysis, but its Type I error rates are sensitive to departures from normality in the trait data. For this reason, the genome-wide significance of the linkage peaks was assessed by simulation. Ten thousand replicates of the chromosome 22 data were simulated using the actual marker map, allele frequencies and missing data patterns, but in the absence of linkage. Thus, in the simulated data sets, alleles were randomly transmitted from parents to offspring, subject to the recombination probabilities dictated by the marker map. Quantitative trait linkage analysis was performed on each of these replicates using the same trait values observed in the actual data. The average number of linkage peaks reaching a given height per replicate of chromosome 22 was calculated (peaks were defined as local maxima of the multipoint LOD score curve separated by at least 30 cM). Since the genetic length of chromosome 22 is approximately 1/44 of the total length of the autosomal genome in the SNP Consortium map, the approximate number of peaks per genome reaching the required height was obtained by multiplying the average number of peaks per replicate of chromosome 22 by 44. Following Lander and Kruglyak (32), a peak was described as ‘genome-wide significant’ if this number was less than 0.05, and ‘genome-wide suggestive’ if it was less than 1. This simulation procedure was found to give similar results to those obtained by simulating replicates of all 22 autosomal chromosomes (P.A.H., unpublished data) and is computationally simpler.

Relative allelic expression analysis

Individuals that are heterozygous for an SNP in transcribed sequence, and therefore informative for allelic expression analysis, were identified by genotyping genomic DNA. Lymphoblast-derived cDNA from heterozygous individuals was then assayed as two separate RT reactions, alongside the corresponding genomic DNA samples. Allelic expression analysis was performed as described previously (19). Briefly, sequence containing the expressed polymorphism rs1047631 was PCR-amplified from cDNA and genomic DNA under identical conditions, followed by primer extension using SNAPshot chemistry (Applied Biosystems). SNAPshot products were electrophoresed on a 3100 DNA sequencer (Applied Biosystems) and peak heights of allele-specific extended primers determined using Genotyper 2.5 software (Applied Biosystems). The ratio of peak heights from cDNA, corrected using the average genomic ratio from all heterozygous individuals, was used to calculate relative expression of the two alleles in each individual sample.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

Conflict of Interest statement. None declared.

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REFERENCES


