ORIGINAL ARTICLE

Identification in 2 Independent Samples of a Novel Schizophrenia Risk Haplotype of the Dystrobrevin Binding Protein Gene (*DTNBP1*)

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Context: Recent research suggests that variation in the gene encoding dystrobrevin binding protein (*DTNBP1*) confers susceptibility to schizophrenia. Thus far, no specific risk haplotype has been identified in more than 1 study.

Objectives: To confirm *DTNBP1* as a schizophrenia susceptibility gene, to identify and replicate specific risk and protective haplotypes, and to explore relationships between *DTNBP1* and the phenotype.

Design: Genetic association study based on mutation detection and case-control analysis.

Setting: All subjects were unrelated and ascertained from general (secondary care) psychiatric inpatient and outpatient services.

Participants: The Cardiff, Wales, sample included 708 white subjects from the United Kingdom and Ireland (221 females) who met *DSM-IV* criteria for schizophrenia and were individually matched for age, sex, and ethnicity to 711 blood donor controls (233 females). Mean ± SD age at first psychiatric contact for cases was 23.6 ± 7.7 years; mean age at ascertainment was 41.8 ± 13.5 years. The Dublin, Ireland, sample included 219 white subjects from the Republic of Ireland who met *DSM-III-R* criteria for schizophrenia or schizoaffective disorder and 231 controls. The mean age of the Irish cases was 46.0 ± 8.5 years; mean age at first psychiatric contact was 25.2 ± 12.4 years.

Main Outcome Measure: Evidence for association between the *DTNBP1* locus and schizophrenia.

Results: In the Cardiff sample, there was no evidence for association with previously implicated haplotypes but strong evidence for association with multiple novel haplotypes. Maximum evidence was found for a novel 3-marker haplotype (global $P<.001$), composed of 1 risk haplotype ($P=.01$) and 2 protective haplotypes, 1 common ($P=.006$) and 1 rare ($P<.001$). Specific risk and protective haplotypes were replicated in the Dublin sample ($P=.02$, .047, and .006, respectively). The only phenotypic variable associated with any haplotype was between the common protective haplotype and higher educational achievement ($P=.02$, corrected for multiple tests).

Conclusions: *DTNBP1* is a susceptibility gene for schizophrenia. Specific risk and protective haplotypes were identified and replicated. Association with educational achievement may suggest protection mediated by IQ, although this needs to be confirmed in an independent data set.

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SCHIZOPHRENIA IS A COMMON disorder with a lifetime morbidity risk of 1%, more if spectrum disorders are included.[1] There is a large genetic epidemiological literature showing that individual differences in liability are largely genetic, the heritability is approximately 80%,[2] and it is a complex genetic disorder, although the precise mode of inheritance is unknown.[2] More than 20 genome-wide linkage scans have revealed several promising linkage findings,[3] and of these, one of the best-supported regions is 6p24-22.[4-8] To identify the specific gene(s) responsible, Straub and colleagues[9] have recently undertaken detailed linkage disequilibrium (LD) across the linked regions of 6p22 in their sample of Irish families in whom linkage was initially observed. Significant association was found between schizophrenia and several individual markers and haplotypes (DNA sequence defined by multiple polymorphic sites) across the gene encoding dystrobrevin binding protein (*DTNBP1*).[10] Although the findings suggested *DTNBP1* as a susceptibility gene for schizophrenia, Straub and colleagues were unable to identify the specific susceptibility variants in the original analysis or in a reanalysis of the same sample based on extra marker information.[11]

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A follow-up study of *DTNBP1* was performed based on 78 German and Israeli families who showed evidence for linkage to 6p and 127 proband-parent trios, mainly from Germany but including a small number of subjects from Hungary.[12] Once again, evidence for association was obtained with individual markers, a finding that was strengthened by analysis of haplotypes. Although the same haplotype was independently associated in each of the 2 samples (linkage families and trios), it was defined by the most common allele at each of the 6 markers examined, a finding that contrasts with the earlier study[10] in which the risk haplotype was defined by the least common allele at all loci. Nevertheless, taken with the initial report, the data are consistent with the hypothesis that *DTNBP1* is a susceptibility gene for schizophrenia.

Both of the previous studies were based on a family-based association design. Here, association is detected by finding that a marker or haplotype is transmitted by parents to their affected offspring more often than would be expected by chance (0.5). Rarely (for example, if particular combinations of markers are not favorable to survival), markers may show segregation distortion in family-based studies that relates to some aspect of ascertainment unrelated to the phenotype (in this example, simply being alive) rather than true association. The authors of the first study reported that this was not a likely explanation for their data, because no excess transmission was noted for one of the markers to unaffected offspring, but it would be reassuring to find additional evidence for association in case-control study designs in which segregation distortion does not apply. However, the first published case-control study based on 219 Irish cases and 231 Irish controls failed to find evidence for association between *DTNBP1* and schizophrenia.[13] Failures to replicate are to be expected for loci of small effect, especially when the individual susceptibility variants are unknown, and single reports of this nature do not amount to rejection of the hypothesis. Nevertheless, further support, preferably including case-control data, is required to make the case for *DTNBP1* as a susceptibility gene for schizophrenia unassailable.

In this study, we have used a large case-control sample (708 cases and 711 controls) to seek confirmation of association between *DTNBP1* and schizophrenia by typing the most informative set of markers reported by Straub and colleagues. In addition, we attempted to identify a specific nucleotide variant or variants that confer susceptibility by screening all known and predicted exons of *DTNBP1* for variants and have tested all the single nucleotide polymorphisms (SNPs) we identified for association with schizophrenia using a DNA pooling approach.[14] Moreover, following up on indirect evidence that there are polymorphisms that influence *DTNBP1* expression,[15] we have screened the putative promoter regions of *DTNBP1* for sequence variants and tested these for evidence for association. We found no evidence for association between schizophrenia and the markers studied previously[10,12] and also failed to replicate either of the reported findings with regard to the specific risk haplotypes. However, when we included novel variants in our analysis, we obtained suggestive evidence for single marker association, and when these were added to some of the previous markers to construct haplotypes, we obtained highly significant evidence for association. When the critical marker that was required to make our sample informative was typed in a sample that previously did not show evidence for association with *DTNBP1*,[13] the specific haplotypes that we observed to be significantly more and less common in cases were again significantly associated with schizophrenia. Our data provide compelling evidence that *DTNBP1* is indeed a susceptibility gene for schizophrenia; identify for the first time, to our knowledge, haplotypes that replicate across independent samples; and strongly reject the possibility of segregation distortion confounding the previous family-based studies. Finally, exploration of the relationship between genetic variation in *DTNBP1* and the phenotype of schizophrenia suggests that the common protective haplotype is associated with higher educational attainment. The latter is a crude index of general intelligence (which was not directly assessed), and we therefore postulate that variation *DTNBP1* may modulate risk of schizophrenia by influencing broadly defined cognitive ability.

METHODS

SAMPLES

The Cardiff, Wales, case-control sample consisted of 708 subjects with schizophrenia from the United Kingdom and Ireland (478 males and 221 females) matched for age, sex, and ethnicity to 711 blood donor controls (478 males and 233 females). Of the cases, 141 had at least 1 affected first-degree relative whose diagnosis had been confirmed by identical methods to that of the proband. The samples of familial cases (n=141; 47 females and 94 males; mean±SD age, 49.1±12.9 years) were all ascertained for a sib-pair linkage study,[16] which revealed no evidence for linkage to 6p,[17] with analyses in the region yielding maximum logarithm of odds (LOD) scores of less than 0.4.[18] All patients had a consensus diagnosis of schizophrenia according to *DSM-IV* criteria made by 2 independent raters following a semistructured interview by trained psychiatrists or psychologists using the Schedules for Clinical Assessment in Neuropsychiatry interview[19] and review of case records. The operational criteria checklist (OPCRIT) and global assessment scale (GAS) were also completed.[20,21] High levels of reliability ($\kappa > 0.8$) were achieved between raters for diagnoses and rating scale items. The mean±SD age at first psychiatric contact for the sample was 23.6±7.7 years, and the mean age at ascertainment was 41.8±13.5 years.

The Dublin, Ireland, case-control sample consisted of 219 cases and 231 controls from the Republic of Ireland. All cases were interviewed by a psychiatrist or psychiatric nurse trained to use the Structured Clinical Interview for *DSM*. Diagnosis was based on *DSM-III-R* criteria using all available information (interview, family or staff report, and medical record review). All cases were older than 18 years, were of Irish origin, and met criteria for schizophrenia or schizoaffective disorder. The control sample, obtained from Irish blood donors, was not specifically screened for psychiatric illness, but individuals were not taking regularly prescribed medications. In neither country are blood donors remunerated even for expenses. The mean±SD age of the Irish sample was 46.0±8.5 years: 45.1±13.1 years and 46.9±10.4 years for males and females, respectively. Mean age at first psychiatric contact for cases was 25.2±12.4 years.

For both samples, all cases were screened to exclude substance-induced psychotic disorder or psychosis due to a general medical condition. Ethics committee approval was ob-

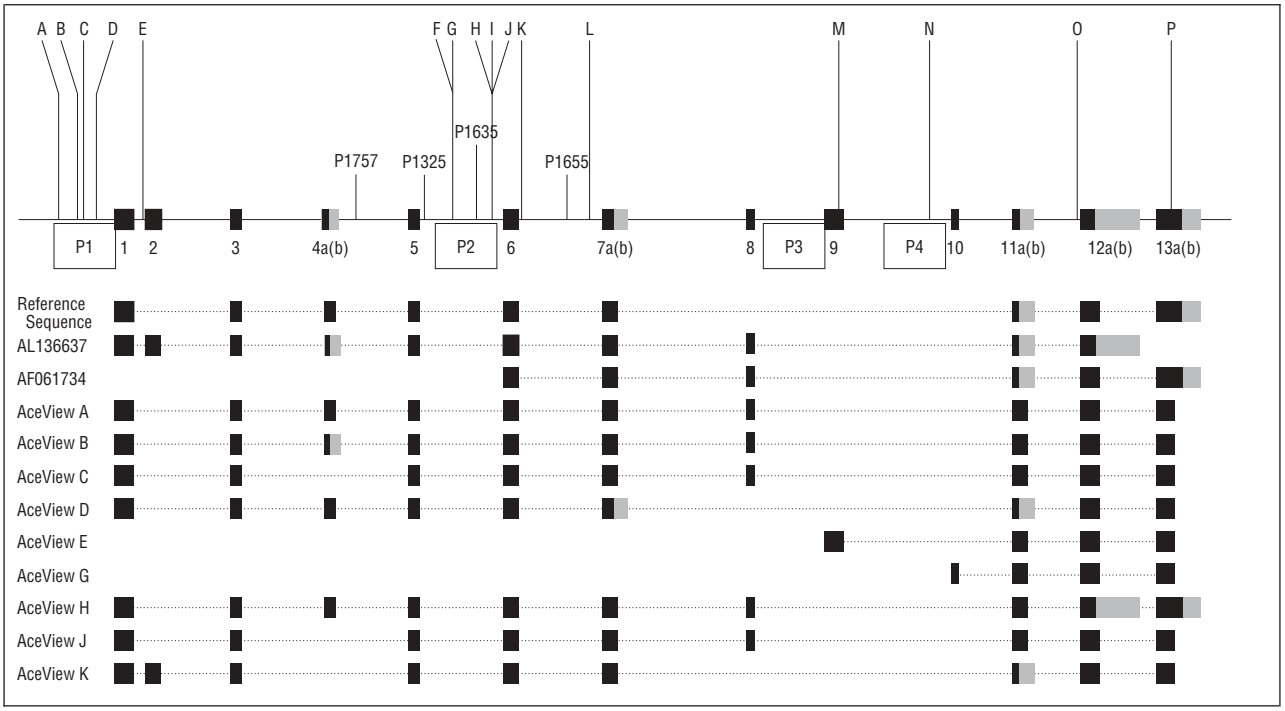


Figure 1. The exonic structure of *DTNBP1* together with that of each transcript used in its construction (not to scale) and the relative locations of the single nucleotide polymorphisms we genotyped that were previously reported[10] together with those identified in this study (A-P) are presented. The shaded areas of exons 4, 7, 11, 12, and 13 represent alternative splicing. P1, P2, P3, and P4 represent the proposed locations of promoters 1, 2, 3, and 4, respectively. AceView is an integrated view of human genes as reconstructed by alignment of all publicly available messenger RNA and expressed sequence tags on the genome sequence (available at:http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?c=locusid&l=84062).

tained in all regions where patients were recruited, and informed written consent was obtained from all participants.

PHENOTYPE ANALYSIS

Phenotype analysis was performed using the Cardiff sample for the main common risk and protective haplotypes. We examined (1) age of onset, defined as the age at which psychiatric help for psychotic symptoms was first sought; (2) severity, defined by worst GAS score; (3) course of illness (ranging from single episode with full recovery to continuous illness with gradual deterioration); and (4) symptom dimensions obtained by factor analysis of OPCRIT psychosis items. As previously observed in a smaller sample,[22] principal components analysis of the OPCRIT psychosis items using direct oblimin rotation resulted in 4 factors being extracted after examination of both eigenvalues and the scree plot. The 4 factors correspond to OPCRIT items regarding paranoid delusions and hallucinations, disorganized symptoms, negative symptoms, and first-rank delusions. Given that low cognitive ability is a risk factor for schizophrenia,[23] we also investigated for association with level of education achieved. This was assessed by the self-declaration of the highest level of qualifications attained. Subjects with no qualifications were scored 0, those with qualifications obtained at school up to but excluding A level (a university entry examination in the United Kingdom) were scored 1, and those with A levels and any further educational qualification were scored 2. Dividing subjects into more categories (for example, affording higher scores for graduate and postgraduate degrees) yielded similar results to the analyses presented in this article.

STATISTICAL ANALYSIS

Of the 8 SNPs that showed significant associations in the study by Straub et al[10], we restricted our analysis to SNPs P1655, P1635, P1325, and P1757. We excluded the other SNPs because they were in very strong LD ($D'>0.9$ and $r^2>0.9$), with at least 1 of the genotyped SNPs.[10] Tests for single-marker allelic association in our case-control sample were performed by χ^2 analysis. Tests for haplotype association with schizophrenia were performed using the software EHPLUS,[24] and statistical significance was estimated using the permutation test PMPLUS.[25] Associations between haplotypes and age at onset, GAS score, and symptom dimensions were analyzed using linear regression models. Course of illness and education were analyzed using ordinal regression.

MUTATION SCREENING

The genomic structure of all known spliced forms of *DTNBP1* (**Figure 1**) was determined *in silico* by combining the reference exonic sequence in the online genetic databases LocusLink and Ensembl (available at: http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=84062/ and http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000047579) with the 12 *DTNBP1* transcripts in AceView (an integrated view of human genes as reconstructed by alignment of all publicly available messenger RNA and expressed sequence tags on the genome sequence, available at: http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?c=locusid&l=84062). All available exons were aligned according to the University of California, Santa Cruz, human genome reference sequence (July 2003 freeze [the version of the genomic sequence that this analysis was based on]) using BLAST 2 sequences[26] (an Internet-based program to perform gapped alignments between DNA sequences) (available at: http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and the genomic structure and sequence used to design amplicons for mutation discovery across all exons.

There are 4 predicted alternative first exons, indicating that it is likely there are 4 independent promoters (Figure 1). Only promoters 1, 2, and 4 were found to contain a characteristic

promoter sequence as determined *in silico* using the software Cister: Cis-element Cluster Finder[27] (available at http://zlab.bu.edu/~mfrith/cister.shtml). However, we screened 2 kb of sequence upstream of the predicted start of transcription of each of the 4 potential promoters.

The derived genomic sequences were used to design primers using Primer3 software (available at: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).[28] Large exons were amplified using sets of amplimers of no more than 600 bases that overlapped by no less than 50 bases. All polymerase chain reactions (PCRs) were performed using standard touchdown protocols previously described.[29] (Details of the PCR conditions are available from the authors.)

The sample for mutation screening consisted of 14 unrelated white subjects from the United Kingdom who met *DSM-IV* criteria for schizophrenia, each of whom had at least 1 affected sibling. The PCR products from each were screened for sequence variation by denaturing high-performance liquid chromatography using a sensitive protocol we have described elsewhere.[29,30] The PCR products from individuals showing chromatograms suggestive of heteroduplex formation were sequenced in both directions using Big-Dye terminator chemistry and an ABI3100 sequencer according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif). All variants were confirmed by allele-specific primer extension using SNaPshot reagents and an ABI3100 sequencer according to the manufacturer's instructions (Applied Biosystems).

GENOTYPING

In Cardiff, individual genotyping was performed by means of single nucleotide primer extension using either the Acycloprime (Perkin Elmer Life Science Products, Boston, Mass) or SNaPshot methods according to manufacturer's instructions, with alleles being determined by fluorescence polarization measurement using an Analyst (LJL Biosystems Ltd, Surrey, England) or an ABI3100 sequencer, respectively. In Dublin, the Irish sample was genotyped using SNaPshot and an ABI377 DNA Sequencer (Applied Biosystems). All polymorphisms that we identified by denaturing high-performance liquid chromatography were genotyped by primer extension in DNA pools constructed from a subset of our cases (n=552) and controls (n=552) taken from the first case-control sample. Analysis was performed on 6 different DNA pools, each containing a different set of 184 cases or controls. Pools were created from DNA that had been quantified using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, Ore) and a Labsystems Fluoroskan Ascent (LifeSciences International, Basingstoke, Hampshire, England) fluorometer. Each DNA pool was amplified in 2 separate PCR reactions and the products subjected to allele-specific primer extension using SNaPshot as described.[14] Estimated allele frequencies were converted to numbers and were tested for approximate statistical significance by χ^2 analysis. Any differences where $P<.10$ were then confirmed by individual genotyping.

LD ANALYSIS

We individually genotyped 96 individuals from the first case-control sample for all SNPs to estimate marker-marker LD. The program ldmax (http://www.sph.umich.edu/csg/abecasis/GOLD/)[31] was used to reconstruct haplotypes and calculate D′ and r^2. An α version of the program Haploview (developed in and maintained by Mark Daly's lab at the Whitehead Institute, Cambridge, Mass, by Jeffrey Barrett; software available for download at http://www-genome.wi.mit.edu/personal/jcbarret/haploview/) was also used to reconstruct haplotypes and to examine haplotype block structure.

Table 1. Tests for Association With 4 Markers*

SNP and Allele	Controls		Cases		χ^2	*P* Value
	Count	Frequency	Count	Frequency		
P1655						
G	682	0.49	679	0.49	0.013	.91
C	716	0.51	719	0.51		
P1635						
G	151	0.11	148	0.11	0.053	.82
A	1251	0.89	1192	0.89		
P1325						
T	127	0.09	139	0.10	0.785	.38
C	1261	0.91	1231	0.90		
P1757						
A	272	0.19	258	0.19	0.208	.64
G	1134	0.81	1124	0.81		

Abbreviation: SNP, single nucleotide polymorphism.
*Markers were selected from Straub et al.[10]

Table 2. Results for the Global Tests for Association for All Haplotype Combinations in Case-Control Sample 1

Haplotype	No. of Cases/Controls	*P* Value
1655-1635-1325-1757	645/677	.22
1655-1325-1757	647/682	.52
1655-1325-1757	676/683	.45
1655-1635-1757	656/691	.41
1655-1635-1325	652/678	.41
1325-1757	678/688	.90
1635-1757	659/696	.81
1655-1757	688/697	.99
1635-1325	655/685	.57
1655-1325	683/684	.81
1655-1635	666/692	.15

RESULTS

The results we obtained in the Cardiff case-control sample for SNPs P1655, P1635, P1325, and P1757[10] are presented in **Table 1**. Only minor differences were found between cases and controls in allele (Table 1) or genotype (not shown) frequencies, none of which approached statistical significance. All makers were in Hardy-Weinberg equilibrium. No further evidence for association was obtained when the markers were used to construct haplotypes (**Table 2**). The specific haplotype implicated by Straub and colleagues,[10] corresponding to GGCA at markers 1655-1635-1325-1757, respectively, was not significantly in excess in cases (0.097 in cases, 0.102 in controls, $\chi^2_1 = 0.186$, $P = .67$). More recently, it has been shown that the risk haplotype in that sample can be fully defined by alleles GCA at the latter 3 loci.[11] In our sample, that specific haplotype was also not significantly more frequent in cases and controls (10.0%: 10.1%, $\chi^2_1 = 0.004$, $P = .95$). Similarly, we did not find the haplotype implicated by Schwab and colleagues[12] (corresponding to alleles ACG at markers 1635-1325-1757) to be significantly in excess in patients (0.71 in cases, 0.71 in controls).

Table 3. Pooled Analysis of 522 Schizophrenic Cases and Controls of SNPs Detected by Denaturing High-Performance Liquid Chromatography in and Around *DTNBP1**

SNP	Position	SNP	Location	Allele Frequency		*P* Value
				Cases	Controls	
A	15773188	A/T	Promoter 1	0.55	0.60	**.06**
B	15773184	C/T	Promoter 1	0.81	0.83	.21
C	15772743	C/G	Promoter 1	0.10	0.13	**.03**
D	15772392	C/T	Promoter 1	0.14	0.16	.12
E	15768850	A/G	Intron 1	0.60	0.63	.26
F	15736802	T/C	Promoter 3	0.25	0.27	.46
G	15736727	A/G	Promoter 3	0.27	0.28	.18
H	15736141	C/T	Promoter 3	0.07	0.06	.37
I	15736060	C/T	Promoter 3	0.15	0.13	.18
J	15735750	G/A	Intron 5	0.86	0.89	**.03**
K	15735532	G/C	Intron 6	0.85	0.86	.50
L	15723616	C/T	Intron 6	0.16	0.15	.62
M	15694111	C/T	Exon 9 (5′ UTR)	0.86	0.87	.53
N	15658414	Gins/del	Promoter 4	0.04	0.04	.69
O	15632897	A/G	Intron 11	<0.05	<0.05	. . .
P	15631080	A/G	Exon 13 (3′ UTR)	0.14	0.14	.96

Abbreviations: del, deletion; *DTNBP1*, dystrobrevin binding protein gene; SNP, single neucleotide polymorphism; UTR, untranslated region.
*SNP positions are according to the University of California, Santa Cruz, human genome reference sequence (July 2003 freeze). SNPs A, C, E, L, and P correspond to rs2619538, rs2743852, rs909706, rs3829893, and rs1047631, respectively. Boldface indicates significant value.

Table 4. Individual Genotyping Results of the SNP Providing Positive Results by Pooling (A, C, and J) and Those Located Within *DTNPB1* Exonic Sequence (M and P)

SNP and Allele	Controls		Cases		*P* Value
	Count	Frequency	Count	Frequency	
A					
T	526	0.47	518	0.43	.06
A	586	0.53	676	0.57	
C					
G	97	0.08	126	0.10	.09
C	1091	0.92	1114	0.90	
J					
A	154	0.12	129	0.10	.06
G	1150	0.88	1219	0.90	
M					
C	174	0.13	156	0.12	.27
T	1124	0.87	1148	0.88	
P					
G	155	0.12	177	0.14	.30
A	1113	0.88	1125	0.86	

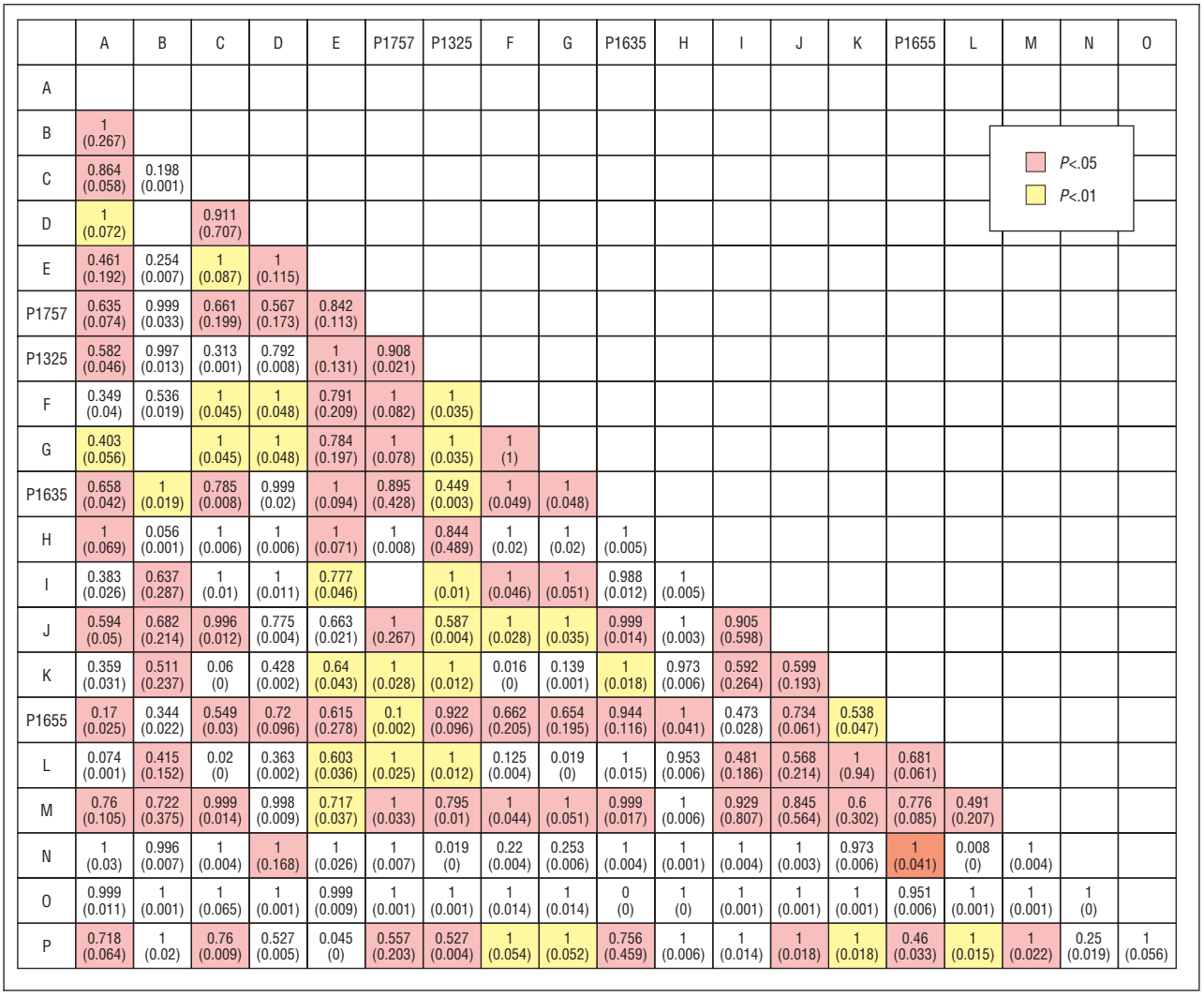
Abbreviation: SNP, single nucleotide polymorphism.

DTNBP1 is predicted to have 13 exons and has at least 12 different known messenger RNA transcripts (Figure 1). We initially screened 6669 bases of genomic sequence of which 3161 were exonic. Of those 3161 bases, 1077 encode amino acids, and 495 and 1589 bases represent the 5′ and 3′ untranslated regions, respectively. The remaining 3508 base pairs were intronic. We found 7 SNPs, 2 of which were exonic but untranslated, whereas the other 5 were in introns. All are aligned with the genomic structure in Figure 1 and listed in **Table 3**. None of the changes are predicted to alter the amino acid sequence of the protein.

Given that we previously obtained evidence that an unknown polymorphism affected *DTNBP1* expression in the cerebral cortex,[15] we extended our mutation screen to the 4 putative promoter regions of *DTNBP1*, because promoters are among (though are not the only) the most important regulatory elements in a gene. A total of 7920 bases of putative promoter sequence were screened, resulting in a further 9 SNPs.

The details of each SNP together with allele frequencies determined from the case-control pools are presented in Table 3. Three SNPs had a difference in allele frequency of $P<.10$, and these were taken to individual genotyping in the Cardiff case-control sample. Given their exonic position, we also genotyped the 2 untranslated region SNPs, despite the fact that neither had shown evidence for association on pooled genotyping. The individual genotype data are similar to those obtained from the pooled genotyping and provide only nonsignificant trends for association (**Table 4**).

We then constructed the 9-marker haplotype that was composed of all the markers we had individually genotyped. This revealed modest evidence for association (global $P=.045$). Five of the 9 possible 8-marker haplotypes also yielded globally significant evidence for association, as did 20 of 36 of the 7-marker haplotypes and 39 of 84 of the 6-marker haplotypes. In total, approximately one third of all possible haplotypes revealed results that gave global significance at $P<.05$. That a high proportion of haplotypes was associated is not surprising given the nonindependence of both individual markers and haplotypes (**Figure 2**), and Bonferroni correction is clearly not appropriate. Marker combinations yielding global evidence for association at $P<.003$ are presented in **Figure 3**. The haplotypes that yielded global evidence for significant association at this level include SNP A in promoter 1 and at least 1 of P1635 and P1655. The haplotype that yielded the strongest global evidence for association consisted of markers P1655, P1635, and SNP A ($\chi^2=31.19$, empirical $P<.001$, 100 000 simulations). The frequencies of each of the



	A	B	C	D	E	P1757	P1325	F	G	P1635	H	I	J	K	P1655	L	M	N	O
A																			
B	1 (0.267)																		
C	0.864 (0.058)	0.198 (0.001)																	
D	1 (0.072)		0.911 (0.707)																
E	0.461 (0.192)	0.254 (0.007)	1 (0.087)	1 (0.115)															
P1757	0.635 (0.074)	0.999 (0.033)	0.661 (0.199)	0.567 (0.173)	0.842 (0.113)														
P1325	0.582 (0.046)	0.997 (0.013)	0.313 (0.001)	0.792 (0.008)	1 (0.131)	0.908 (0.021)													
F	0.349 (0.04)	0.536 (0.019)	1 (0.045)	1 (0.048)	0.791 (0.209)	1 (0.082)	1 (0.035)												
G	0.403 (0.056)		1 (0.045)	1 (0.048)	0.784 (0.197)	1 (0.078)	1 (0.035)	1 (1)											
P1635	0.658 (0.042)	1 (0.019)	0.785 (0.008)	0.999 (0.02)	1 (0.094)	0.895 (0.428)	0.449 (0.003)	1 (0.049)	1 (0.048)										
H	1 (0.069)	0.056 (0.001)	1 (0.006)	1 (0.006)	1 (0.071)	1 (0.008)	0.844 (0.489)	1 (0.02)	1 (0.02)	1 (0.005)									
I	0.383 (0.026)	0.637 (0.287)	1 (0.01)	1 (0.011)	0.777 (0.046)		1 (0.01)	1 (0.046)	1 (0.051)	0.988 (0.012)	1 (0.005)								
J	0.594 (0.05)	0.682 (0.214)	0.996 (0.012)	0.775 (0.004)	0.663 (0.021)	1 (0.267)	0.587 (0.004)	1 (0.028)	1 (0.035)	0.999 (0.014)	1 (0.003)	0.905 (0.598)							
K	0.359 (0.031)	0.511 (0.237)	0.06 (0)	0.428 (0.002)	0.64 (0.043)	1 (0.028)	1 (0.012)	0.016 (0)	0.139 (0.001)	1 (0.018)	0.973 (0.006)	0.592 (0.264)	0.599 (0.193)						
P1655	0.17 (0.025)	0.344 (0.022)	0.549 (0.03)	0.72 (0.096)	0.615 (0.278)	0.1 (0.002)	0.922 (0.096)	0.662 (0.205)	0.654 (0.195)	0.944 (0.116)	1 (0.041)	0.473 (0.028)	0.734 (0.061)	0.538 (0.047)					
L	0.074 (0.001)	0.415 (0.152)	0.02 (0)	0.363 (0.002)	0.603 (0.036)	1 (0.025)	1 (0.012)	0.125 (0.004)	0.019 (0)	1 (0.015)	0.953 (0.006)	0.481 (0.186)	0.568 (0.214)	1 (0.94)	0.681 (0.061)				
M	0.76 (0.105)	0.722 (0.375)	0.999 (0.014)	0.998 (0.009)	0.717 (0.037)	1 (0.033)	0.795 (0.01)	1 (0.044)	1 (0.051)	0.999 (0.017)	1 (0.006)	0.929 (0.807)	0.845 (0.564)	0.6 (0.302)	0.776 (0.085)	0.491 (0.207)			
N	1 (0.03)	0.996 (0.007)	1 (0.004)	1 (0.168)	1 (0.026)	1 (0.007)	0.019 (0)	0.22 (0.004)	0.253 (0.006)	1 (0.004)	1 (0.001)	1 (0.004)	1 (0.003)	0.973 (0.006)	1 (0.041)	0.008 (0)	1 (0.004)		
O	0.999 (0.011)	1 (0.001)	1 (0.065)	1 (0.001)	0.999 (0.009)	1 (0.001)	1 (0.001)	1 (0.014)	1 (0.014)	0 (0)	1 (0)	1 (0.001)	1 (0.001)	1 (0.001)	0.951 (0.006)	1 (0.001)	1 (0.001)	1 (0)	
P	0.718 (0.064)	1 (0.02)	0.76 (0.009)	0.527 (0.005)	0.045 (0)	0.557 (0.203)	0.527 (0.004)	1 (0.054)	1 (0.052)	0.756 (0.459)	1 (0.006)	1 (0.014)	1 (0.018)	1 (0.018)	0.46 (0.033)	1 (0.015)	1 (0.022)	0.25 (0.019)	1 (0.056)

Figure 2. D′ values (r^2 values) for all combinations of the 20 single nucleotide polymorphisms spanning the *DTNBP1* locus included in this study.

individual haplotypes (with frequency >0.01) for this combination of markers in cases and controls are given in **Table 5**. Post hoc inspection of the individual haplotypes shows that those consisting of alleles CAT are significantly in excess in our cases, whereas those consisting of CAA and GGT are significantly more common in controls.

To attempt to replicate our data, we analyzed the Dublin case-control sample that did not support association when analyzed by the previously available markers.[13] The haplotype frequencies in this sample are remarkably similar to the Cardiff sample (Table 5), and the same CAT and the CAA and GGT haplotypes were significantly more and less common, respectively, in the schizophrenic cases. On this occasion, analyses of the individual haplotypes were under the specific hypotheses generated from the first sample rather than post hoc, and therefore although the *P* values obtained were more modest (in keeping with the sample size), their prior probability is greater. When we combine the samples, the specific haplotypes CAT, CAA, and GGT give highly significant evidence in favor of association ($\chi^2=10.42$, $P=.001$; $\chi^2=10.80$, $P=.001$; $\chi^2=35.96$, $P<.001$, respectively). The estimated odds ratio for the CAT risk haplotype is 1.40 (95% confidence interval, 1.13-1.74).

Estimates of LD between all of the SNPs identified in this study and those associated with schizophrenia are presented in Figure 2.

We performed 8 tests for phenotype association with 2 haplotypes, a total of 16 tests. Analysis of the CAT (risk) haplotype revealed no significant association with any aspect of the phenotype. Age was associated with the CAA (protective) haplotype in cases but not in the controls. Although this is almost certainly a spurious finding, it was necessary to adjust for age in the phenotype analyses. Analysis of the CAA protective haplotype revealed a significant association only with higher educational attainment (likelihood ratio test $\chi^2_1=10.6$, $P=.001$, corrected $P=.02$). Although there was no evidence for age at onset as a confounder, this analysis was restricted to subjects with age at onset of younger than 21 years to remove any influence of early onset on education. Without this restriction, evidence for association remained (likelihood ratio test $\chi^2_1=6.6$, $P=.01$).

COMMENT

Using a large case-control sample, we attempted to replicate and extend the original[10] and the subsequent[12] reports implicating *DTNBP1* as a susceptibility gene for

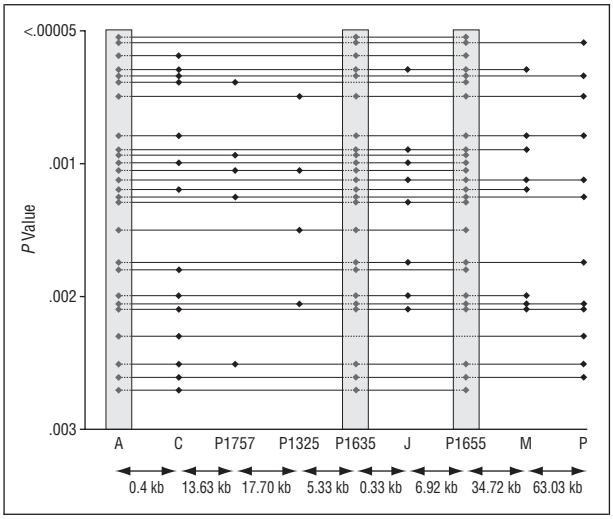


Figure 3. Plot of haplotypes showing global association to schizophrenia ($P<.003$). The most significant result is the 3-marker haplotype composed of single nucleotide polymorphisms (SNPs) A, P1635, and P1655 ($P = .000056$). As highlighted, all other significant haplotypes contain SNP A and either P1635 and/or P1655. The x-axis scale is nonlinear in order to allow easy visualization of the different haplotypes. The intermarker distances are given in kilobases (kb).

schizophrenia. Analyses using the most significant and informative SNPs from those studies revealed no evidence for allelic, genotypic, or haplotype association. Moreover, despite using a screening sample with power of 0.8 to identify variants with a frequency of 0.05, sequence analysis of the exons revealed no nonsynonymous variants in the coding sequence that might account for previous findings. It may be relevant that the previous studies included samples that contributed to positive linkage signals on 6p, whereas our samples did not.[18]

However, following up our report of polymorphic, *cis*-acting influences on *DTNBP1* expression, we screened 4 putative *DTNBP1* alternative promoters for sequence variants. Pooled genotyping of the SNPs provided modest evidence for allelic association for 3 SNPs, 2 of which are located in putative promoters. Although individual genotyping in an extended sample did not yield evidence for allelic association, inclusion of these SNPs, particularly SNP A, with the previous markers yielded strong evidence for association. Moreover, we were able to exactly replicate the pattern of our findings in a completely independent sample. Because our primary data set is case control, our findings cannot be attributed to segregation distortion. The data from the 2 samples we report in this study when viewed in the context of the 2 previous studies[10,12] form an impressive and convincing body of evidence that variation at the *DTNBP1* locus confers susceptibility to schizophrenia. Moreover, we also demonstrate that this effect generalizes to samples that do not display evidence for linkage to 6p. Although more genetic studies will be required before we understand precisely how genetic variation at this locus confers susceptibility, there is now sufficient evidence to justify intensive investigations into disease mechanisms. Our data suggest the existence in our samples of a single risk haplotype defined by CAT, several neutral haplotypes, and 2 protective haplotypes (CAA and GGT). The GGT haplotype was not observed in any schizophrenic subjects and may therefore represent a protective factor that is rare but of substantial effect. Identifying the mechanisms underlying such a strong protective effect may offer an important opportunity for therapeutic intervention.

Although our data from both samples are internally consistent, our risk haplotype has no overlap with that in the original Irish sample.[10,11] Our risk haplotype carries allele A at P1635 like the (largely) German sample,[12] so it is possible that our markers offer a more powerful definition of a haplotype that is common to both populations. However, this can only be established if the German group types our additional critical markers.

We attempted to identify particular aspects of the phenotype that are associated with the 2 common risk and protective haplotypes. Given that *DTNBP1* is thought to be a member of the dystrophin protein complex (DPC),[32] that mutations in the dystrophin gene can result in lower IQ,[33] and that low cognitive ability is a risk factor for schizophrenia,[23] we also sought evidence for association with educational achievement. We used this as a proxy for general cognitive ability. Although educational achievement is only a crude measure of IQ, there is evidence for correlation ($r=0.4$-0.6) between the 2 measures.[34] Our finding that higher educational achievement is associated with the protective haplotype suggests the *hypothesis* that the protective haplotype of *DTNBP1* modifies the risk of schizophrenia by influencing cognitive ability. Further work will be required to resolve this issue.

Our data also provide a reminder that if allelic heterogeneity occurs in complex diseases, failure to replicate association to specific markers should be treated cautiously. In our study, positive findings only emerged when we generated novel SNPs. We had hoped that by examining 20 SNPs at an average density of 7.5 kb across *DTNBP1*, we would be able to establish the haplotype block structure[35] of the region, because this is likely to facilitate further replication studies, help understanding of the relationship among the different patterns of association among studies, and allow more exact localization of the source(s) of the LD signal. Even at this density, we were unable to formally define any haplotype blocks that have not been subjected to ancestral recombination according to the method of Gabriel and colleagues.[35] Like van den Oord and colleagues,[11] we found a limited number of common haplotypes across dysbindin and many adjacent marker pairs were in strong LD (Figure 2), but our findings differ in that the markers that define the risk haplotype extend beyond the region suggested by that group (corresponding to SNP E-P1635, Figure 1) to include the putative promoter.

Our data do not implicate individual SNPs or haplotypes as acting directly to increase disease susceptibility. Moreover, although we have previously shown that *cis*-acting elements regulate *DTNBP1* expression in native brain tissue,[15] we cannot conclude that this is the mechanism underlying the association. Unfortunately, because the minor allele frequencies of the expressed SNPs are low, much larger tissue sample sizes than we currently have available will be required if allele-specific expression assays are to be used to relate specific haplotypes to gene expression in the way we have done for *COMT*.[36]

Table 5. Frequencies of All Haplotypes for Markers P1655-P1635-SNP66961 in the Cardiff and Dublin Case-Control Samples*

	Cardiff Sample				Dublin Sample			
P1655-P1635-SNP A	Cases (n = 529)	Controls (n = 590)	χ^2_1	*P* Value	Cases (n = 216)	Controls (n = 228)	χ^2_1	*P* Value
GAA	0.13	0.14	0.07	.78	0.11	0.11	. . .	. . .
CAA	0.29	0.35	7.5	.006	0.30	0.37	3.9	.047
GGA	0.10	0.08	3.4	.06	0.10	0.10	. . .	. . .
GAT	0.26	0.24	0.58	.44	0.28	0.26	. . .	. . .
CAT	0.21	0.16	6.16	.01	0.21	0.15	5.7	.02
GGT	0.00	0.03	28.2	<.001	0.00	0.02	7.65	.006

Abbreviation: SNP, single nucleotide polymorphism.
*Haplotypes with frequencies less than 0.01 were excluded. Global *P* value for the Cardiff sample: $\chi^2 = 31.19$, empirical $P<.001$, 100 000 simulations; global *P* value for the Dublin sample: $\chi^2 = 10.92$, empirical $P = .06$, 1000 simulations; and global *P* value combined: $\chi^2 = 40.01$, empirical $P<.001$, 100 000 simulations.

The link between abnormal dysbindin function, whatever the genetic mechanism, and schizophrenia is also unclear. Dysbindin is expressed widely in the brain and other tissues.[10] In the brain, dysbindin binds β-dystrobrevin. β-Dystrobrevin is a member of the DPC found in postsynaptic densities.[32] Straub and colleagues[10] postulated that compatible with the DPC's roles in synaptic structure, maintenance and synaptic signaling, altered dysbindin or DPC function may lead to several of the structural and functional abnormalities that have been reported in schizophrenia, including altered function at glutamatergic and γ-aminobutyric acid synapses and reduced synaptic density in frontal cortex and hippocampus. Recently, it has also been shown that mice that express no dystrophin (and therefore have altered DPC function) have abnormal development of the posterior cerebellar vermis.[37] The authors of that study[37] postulated that similar abnormalities in cerebellar development as a result of altered dysbindin function may result in several other abnormalities that have been reported in schizophrenic patients, including altered working memory, eye movement, and cerebellar structure.

Schizophrenia in particular and psychiatric disorders in general have previously been thought of as relatively impervious to molecular genetic analysis. It is therefore ironic that with the recent positional cloning and subsequent confirmation of *DTNBP1*,[10,12] *NRG1*,[38-40] and possibly *G30/G70*,[41,42] schizophrenia is now one of the few disorders in which genes of small-to-modest effect have been identified by positional genetics. These successes are immensely encouraging. First, the existence of several promising linkages suggests that other susceptibility genes for schizophrenia are likely to be found in the coming years. Second, the identification of novel genes and pathways in the pathogenesis of schizophrenia will open new vistas for neurobiological research[43] and the prospect of improvements in diagnostics and treatment.

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