Analysis of TBX1 Variation in Patients with Psychotic and Affective Disorders

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A significant portion of patients with 22q11 deletion syndrome (22q11DS) develop psychiatric disorders, including schizophrenia and other psychotic and affective symptoms, and the responsible gene/s are assumed to also play a significant role in the etiology of nonsyndromic psychiatric disease. The most common psychiatric diagnosis among patients with 22q11DS is schizophrenia, thought to result from neurotransmitter imbalances and also from disturbed brain development. Several genes in the 22q11 region with known or suspected roles in neurotransmitter metabolism have been analyzed in patients with isolated schizophrenia; however, their contribution to the disease remains controversial. Haploinsufficiency of the TBX1 gene has been shown to be sufficient to cause the core physical malformations associated with 22q11DS in mice and humans and via abnormal brain development could contribute to 22q11DS-related and isolated psychiatric disease. 22q11DS populations also have increased rates of psychiatric conditions other than schizophrenia, including mood disorders. We therefore analyzed variations at the TBX1 locus in a cohort of 446 white patients with psychiatric disorders relevant to 22q11DS and 436 ethnically matched controls. The main diagnoses included schizophrenia (n = 226), schizoaffective disorder (n = 67), bipolar disorder (n = 82), and major depressive disorder (n = 29). We genotyped nine tag SNPs in this sample but did not observe significant differences in allele or haplotype frequencies in any of the analyzed groups (all affected, schizophrenia and schizoaffective disorder, schizophrenia alone, and bipolar disorder and major depressive disorder) compared with the control group. Based on these results we conclude that TBX1 variation does not make a strong contribution to the genetic etiology of nonsyndromic forms of psychiatric disorders commonly seen in patients with 22q11DS.

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step in the conversion of proline to the neurotransmitter glutamate (30). Mouse studies support a role in schizophrenia (31,32), and population studies have found an association of PRODH variants with the disorder (33,34), but overall, evidence from population studies is still controversial (35–37). Catechol-O-methyltransferase (COMT) inactivates catecholamine neurotransmitters (dopamine, noradrenalin, and adrenaline) by transferring a methyl group from S-adenosyl methionine (38). This role has made COMT a prime suspect in the genetic etiology of schizophrenia, but evidence from population studies has failed to provide definitive proof (reviewed in 26,39). A third gene, ZDHHC8, was implicated on the basis of a systematic scan of the 22q11 region in patients with schizophrenia and subsequent mouse studies showing a schizophrenia-related phenotype (16,40). However, subsequent replication studies in schizophrenic patients did not support a role of ZDHHC8 (41–45).

The neurodevelopmental model of schizophrenia is based on evidence for perturbances in brain development (46). The observation that craniofacial and limb anomalies are correlated with schizophrenia supports this hypothesis and suggests the disturbance of a shared morphogenetic mechanism (47,48). The key 22q11DS gene, TBX1, is involved in epithelial/mesenchymal interactions (49,50), a mechanism crucial for the development of a wide variety of organs including the forebrain, heart, face, and limbs (51,52). It is therefore conceivable that TBX1 plays a role in brain development and thus in 22q11DS-associated as well as nonsyndromic psychiatric disease.

Several mouse studies have analyzed Tbx1 expression in the brain and, with one exception (53), have provided convincing evidence for expression at low levels during prenatal brain development and then in a steadily increasing fashion from birth to three months (54–56). In addition, evidence supporting a role of TBX1 in psychiatric disease comes from mouse studies that have analyzed prepulse inhibition (PPI), a measure of sensorimotor gating that is abnormal in 22q11DS (57) as well as in several nonsyndromic psychiatric disorders including schizophrenia, posttraumatic stress disorder (reviewed in 58), Tourette’s syndrome (58–60), and obsessive compulsive disorder (61). The PPI deficits observed in mice harboring large heterozygous deletions of the equivalent of the region commonly deleted in 22q11DS have been shown to be caused in part by haploinsufficiency of Tbx1 (55,62). However, our own study failed to show sensorimotor gating deficits in mice heterozygous for loss of Tbx1 (63).

We tested whether TBX1 variation was associated with psychotic and affective disorders relevant to 22q11DS in a large cohort of white patients. Because psychiatric manifestations seen in 22q11 deletion syndrome include psychotic and affective diagnoses, we included in our analysis a total of 446 samples from patients diagnosed with schizophrenia, schizoaffective disorder, bipolar disorder, major depression, or psychotic and mood disorder not otherwise specified (NOS). To capture the largest amount of haplotypes possible, we determined the haplotype block structure of the TBX1 locus and selected tag SNPs. To detect association of variants that directly influence TBX1 expression levels, we included about 20 kb of the 5′ untranslated region. This interval contains several conserved elements showing high interspecies conservation, including a known functional enhancer (64).

All single-SNP as well as haplotype analyses failed to detect an association, leading us to conclude that TBX1 is not likely to play a major role in the genetic etiology of nonsyndromic psychotic and affective disorders.

MATERIALS AND METHODS

Study Subjects

The study sample comprised 446 white patients residing in the USA (mean age = 36.2 years; 36.2% females). Diagnostic categories included schizophrenia (n = 226), schizoaffective disorder (n = 67), bipolar disorder (n = 82), major depression (n = 29), psychotic disorder NOS (20), and affective disorder NOS (22). The control group consisted of 436 white individuals (mean age = 38.73 years, 52.06% females).

Case patients were recruited from the inpatient and outpatient clinical services of the Zucker Hillside Hospital, a division of the North Shore–Long Island Jewish Health System (NSLIJHS). Inclusion criteria for screening for this study included a clinical diagnosis of a psychotic disorder, no active substance abuse, and ability to provide informed consent. After written informed consent was obtained, each subject was assessed with the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID, version 2.0,8/98), administered by trained raters. Standardized diagnostic assessments were supplemented with clinical information obtained by review of medical records and interviews with family informants when possible, and all diagnostic information was compiled into a narrative case summary. Information on the onset and course of axis I illness, presence of axis II pathology, presence of axis III diagnoses, and a brief description of the subject’s psychosocial and occupational functioning during the course of illness was presented to a consensus diagnostc committee consisting of a minimum of three senior faculty with DSM-IV diagnostic experience, as well as other faculty and trainees with SCID experience. All available information was used to arrive at a consensus DSM-IV diagnosis.

Healthy controls were identified and recruited by the Zucker Hillside Hospital Healthy Control Project. Potential participants were recruited via local newspaper advertisements, flyers, and community Internet resources and underwent initial telephone screening to assess eligibility criteria. Subjects meeting eligibility criteria were administered the SCID-NP to rule out the presence of an axis I psychiatric disorder. A urine toxicology screen was conducted to rule out the use of any
drugs, and a family history questionnaire was conducted to rule out the presence of familial psychiatric disorders. Exclusion criteria included current or past: axis I psychiatric disorder, psychotropic drug treatment, substance abuse, first-degree family member with an axis I psychiatric disorder, or inability to provide written informed consent. A subset of the control cohort was collected through the Massachusetts General Hospital Clinical Research Program in conjunction with the Harvard-Partners Center for Genetics and Genomics in Boston, MA, USA. This subset comprised disease-free subjects older than 18 years. For the purposes of the study, “disease” was defined as current or past diagnoses made by a medical care provider that required medication or other forms of treatment/therapy. With the exception of orthopedic procedures, appendectomy, or trauma-related conditions, surgery was considered an exclusionary criterion. Subjects who took prescription medications or regularly used over-the-counter medications were also excluded. Subjects completed a structured family and medical questionnaire that detailed current and past psychiatric illness and pharmacological or psychotherapeutic psychiatric treatment. All responses to the self-report form were confirmed by clinical interview by physicians. Physical exams were completed at the study visit. This study was approved by the Institutional Review Board at NSLIJHS and Partners Healthcare.

The set of Caucasian control DNAs used to develop tag SNPs was obtained from Coriell (Camden, NJ, USA).

Tag SNP Selection

Forty-four SNPs were validated in a small set of normal controls. All SNPs that were monomorphic or had high failure rates were excluded from further analyses. Nineteen SNPs with a minor allele frequency of >5% across the TBX1 locus were genotyped in a set of 94 Caucasian controls (Coriell). All but one SNP were derived from the dbSNP database.

SNP (v14186) was derived through our own SNP discovery efforts and has not yet been assigned a dbSNP ID. All SNPs that were in Hardy-Weinberg equilibrium were then searched for contiguous subsets with reduced haplotype diversity. Together with custom-designed scripts, we used the programs PMPLUS (66) and EHPLUS (67), the latter of which employs the expectation-maximization algorithm to infer haplotype frequencies in the population. By sliding expanding and contracting windows along the region, we identified blocks of markers for which a large percentage of data were seen to be represented by just a few haplotypes. A reduced set of markers to tag the blocks was selected in such a way as to preserve the observed diversity among the major haplotypes (freq > 0.05). In situations for which more than one set of choices were available, we considered two additional factors: preference was given to markers with lower failure rates; marker subsets were chosen such that the greatest percentage of data.

Figure 1. Linkage disequilibrium (LD) at the TBX1 locus and location of SNPs. The genomic organization of the TBX1 gene is shown above. Exons 1-9A, 9B, and 10 are symbolized by vertical bars. The star represents a known enhancer (64). Below, the underlying LD structure is shown. LD strength is symbolized by shades of red (the darker the higher). Solid black lines show three haplotype blocks. SNP identification numbers are listed on the right. SNPs selected for genotyping cases and controls are circled.
could be pooled into the major haplotypes as defined by the tags.

**SNP Genotyping**

Multiplex PCR was carried out to generate short PCR products (>100 bp) containing one SNP or insertion deletion. Briefly, 2.5 ng genomic DNA was amplified in a 5 µL reaction containing 1 x HotStar Taq PCR buffer (Qiagen, Valencia, CA, USA), 2.5 mM MgCl₂, 200 µM each dNTP, 50 nM each PCR primer, and 0.1 U HotStar Taq (Qiagen). The reaction was incubated at 95 °C for 15 min followed by 45 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 1 min, followed by 3 min at 72 °C. Excess dNTPs were then removed from the reaction by incubation with 0.3 U shrimp alkaline phosphatase (USB) at 37 °C for 20 min followed by 5 min at 85 °C to deactivate the enzyme. Single primer extension over the SNP or insertion/deletion was carried out in a final concentration of 600 nM each extension primer, 50 µM d/ddNTP and 0.126 U Thermosequenase (Solis Biodyne, Tartu, Estonia) and incubated at 94 °C for 2 min followed by 45 cycles of 94 °C for 5 s, 52 °C for 5 s, and 72 °C for 5 s. The reaction was then desalted by addition of a cation exchange resin followed by mixing and centrifugation to settle the contents of the tube. The extension product was then spotted onto a 384-well spectrCHIP before being flown in the MALDI-TOF mass spectrometer.

**Statistical Analyses**

Haplotype block structures for the nine tag SNPs were determined in the association study control sample using Haploview version 3.32 (68). Haplotype blocks were defined in accordance with the Gabriel method (69) with a minimum criterion for strong pairwise linkage disequilibrium (D’) between markers set to .90 and default settings for all other criteria. Using this method, three haplotype blocks were identified (Figure 1). Allelic and haplotypic χ² tests were conducted to test for association with any major affective or psychotic disorder (all affects), schizophrenia or schizoaffective disorder (schizophrenia and schizoaffective), schizophrenia only (schizophrenia), and affective disorder only (bipolar disorder and major depressive disorder). Allelic and haplotypic χ² tests were conducted to test for association using the same subgroups.

**RESULTS**

Because this study was initiated prior to completion of the Phase I HapMap, preliminary work was conducted to identify a set of SNPs with comprehensive, yet nonredundant coverage of the TBX1 locus. First, 44 SNPs chosen from dbSNP and our own SNP discovery efforts as well as published studies (65) were validated in a small set of normal control samples. Of these, 19 SNPs with a minor allele frequency of >5% across the TBX1 locus were genotyped in a set of 94 Caucasian controls. One SNP (rs2238775) was not in Hardy-Weinberg equilibrium and was excluded from further analysis. We reanalyzed our data with Haploview (68) because tag SNPs were generated before this now standard software was available. One tag SNP differed between our selection and those selected by Haploview with default settings. However, with a minor change in block definition, the tags selected by Haploview were identical to our initial selection. To define our blocks, we started with those defined by the Gabriel method (69) with the minimum fraction of strong linkage disequilibrium in in-
Formative comparisons set to 0.8 and a minimum haplotype frequency of 5%, and simply extended them to include those flanking SNPs whose inclusion kept the sum of haplotypes captured above 85%. With these methods, we were able to eliminate the need to genotype 10 SNPs in high linkage disequilibrium with our tags, resulting in a final set of nine SNPs genotyped in our set of 446 samples of patients diagnosed with either schizophrenia, schizoaffective disorder, bipolar disorder or major depression, psychotic and mood disorder NOS.

Figure 1 shows the TBX1 genomic structure as well as the location of SNPs in relation to the underlying block structure. Figure 2 shows the pattern of linkage disequilibrium across the nine SNPs in the association sample. Three pairs of SNPs remained in very high linkage disequilibrium and formed haplotypes according to the modified Gabriel criteria described above. None of the nine individual SNPs showed a positive association in the “all affecteds” category (all nominal P values > 0.05), nor did any of the three haplotypes (all nominal P values > 0.05). Similarly, analysis of the individual subgroups of patients compared with healthy controls, (schizophrenia/schizoaffective, n = 293; schizophrenia only, n = 226; affective disorder n = 111) remained entirely negative (Table 1) as did haplotype analysis of the same patient categories (Table 2).

**DISCUSSION**

22q11DS is one of the strongest genetic risk factors for the development of schizophrenia, and the region deleted in patients with 22q11DS is assumed to contain one or several genes that are also involved in the genetic etiology of nonsyndromic schizophrenia. Based on the hypothesis that disturbed brain development contributes to schizophrenia and the fact that haploinsufficiency of TBX1 causes the main developmental anomalies of 22q11DS, we analyzed a possible contribution of TBX1 variation to the genetic etiology of schizophrenia and other nonsyndromic psychiatric disorders seen in patients with 22q11DS.

We genotyped a set of tag SNPs across the TBX1 gene in a set of 446 white patients and 436 ethnically matched controls. Allele and haplotype frequencies did not differ significantly between all affected patients and controls, and no significant differences were observed between cases and controls in the diagnostic subcategories.

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<th>Table 1. Allele frequencies</th>
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Our negative findings can be interpreted several ways. First, TBX1 may not
play a role in the genetic etiology of the nonsyndromic versions of the psychiatric disorders included in this study. This finding would be concordant with results of our own study showing that mice with heterozygous loss of Tbx1 do not show the same sensorimotor gating deficits observed in mice carrying large deletions affecting Tbx1 and 26 other genes (63). The lack of association would also be supported by data published by Hiroi et al who found that although the behavioral anomalies of mice transgenic for a segment containing four genes including TBX1 could be ameliorated by antipsychotic drugs, this effect was not observed in mice heterozygous for TBX1 loss (70). However, our findings contrast with those of a study by Paylor et al (55), who showed that prepulse inhibition (PPI) defects seen in a mouse model of 22q11DS are caused by haploinsufficiency of two genes in the deleted region, Tbx1 and Gnb1l. PPI is a measurable endophenotype of several psychiatric disorders including schizophrenia and can be measured in humans as well as mice. Of note, the mouse studies by Long et al (63) and Paylor et al (55) were both carried out in mice with a mixed genetic background and are difficult to compare because strain-dependent penetrance of the sensorimotor gating deficit cannot be excluded. Alternatively, the lack of TBX1 association in our sample may be a false-negative result. Of note, associations of two genes, DTNBP1 and COMT, have been shown in the same cohort (71,72). Statistical power is a common concern when considering negative results in genetic association studies of complex disease. It is clear that psychiatric illnesses are polygenic, with any single genetic locus likely to contribute only a small proportion of the risk variance (73). Consequently, association studies should be adequately powered to detect effect sizes (odds ratio, OR) of 1.5 or less. Given the sample sizes of cases and controls in the present study, we would have had approximately 80% power to detect a similar effect for TBX1. Furthermore, all TBX1 SNPs and haplotypes examined in the present study would have had power of 70%–80% to detect effects as low as OR = 1.3 for all such variants, and had nearly 100% power to detect ORs > 1.5. Thus, our results are unlikely to be false negatives, and even moderate effects for TBX1 on major psychiatric illness can be excluded. However, it is still possible
that very small effects (1 < OR < 1.3) were not detected.

In summary, it is unlikely that TBX1 plays a major role in the genetic etiology of nonsyndromic schizophrenia and other psychiatric disorders observed in patients with 22q11DS. However, it is important to point out that schizophrenia associated with 22q11DS may represent a genetic subclass that is very similar but genetically distinct from the nonsyndromic form (13,74,75). In this case TBX1 may still contribute to the risk of developing psychiatric disease in patients with 22q11DS.

ACKNOWLEDGMENTS

This project was supported by grants HD034980-09 to RK, K23MH01760 to AKM, K01 MH65580 to TL, P30 MH60575 to JK, and a General Clinical Research Center (M01 RR18335). We thank the HPCGG Genotyping core for their fast and excellent service and Dr. Bernice Morrow for providing services to generate lymphoblastoid cell lines and DNA.

REFERENCES

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