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Neither Single-marker nor Haplotype Analyses Support an Association Between Genetic Variation Near NOTCH4 and Bipolar Disorder

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Markers near the NOTCH4 locus on chromosome 6p21.3 have been reported to be associated with schizophrenia in some studies. Since schizophrenia and bipolar affective disorder (BPAD) may share genetic determinants, we tested markers in and near NOTCH4 in a sample of 153 parent–offspring triads ascertained through a sibling pair with BPAD for evidence of association. This sample would have 80% power to detect an association at or above a genotype relative risk of 2.4 at the 10^{-7} level of significance. In addition to the two markers previously showing the most significant association with schizophrenia, three additional nearby markers were studied. The five markers were genotyped using validated methods. Both single-marker and 3-marker haplotype data was analyzed using family-based association methods. No genome-wide significant association was detected between any of the five SNP-markers and BPAD in this sample. One marker showed nominal evidence of association ($P = 0.049$), but this evidence was not supported by haplotype analyses including nearby flanking markers or by case-control analysis using 93 Caucasian controls. These results do not support an association between genetic variation near NOTCH4 and BPAD in this sample.

KEY WORDS: bipolar disorder; NOTCH4; chromosome 6p21.3; association studies

INTRODUCTION

Bipolar affective disorder (BPAD) is a mood disorder consisting of unpredictable vacillations between mania and clinical depression. Chromosomes 4, 12, 13, 18, 21, 22, X, among others, have been implicated as potential sites for susceptibility loci, but no linkage findings to date have met the most rigorous standards of replication [Prathikanti and McMahon, 2001]. BPAD is a complex genetic disorder most likely involving several genes [Gershon, 2000]; but to date, none have been identified.

Wei and Hemmings [2000] found a strong association between schizophrenia and markers near the *NOTCH4* gene on chromosome 6p21.3, near the major histocompatibility locus (HLA region). They analyzed 13 loci spanning 1.8 Mb of DNA in 80 British parent–offspring trios with schizophrenia. Four loci produced significant evidence of association. These findings are the most statistically significant genetic associations found in psychiatry so far. Since the probability of replication in a study is relative to the strength of the association, these findings should be highly replicable [Suarez, 1994], yet two studies have failed to support any association with schizophrenia [McGinnis et al., 2001; Sklar et al., 2001].

Schizophrenia and BPAD may share genetic determinants. Berrettini [2000] specifically cites genome scan overlaps of linkage findings in BPAD and schizophrenia at 18p11 and 22q11. There is also suggestive evidence of linkage for both schizophrenia and BPAD near

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6q21-22 [reviewed in Nurnberger and Foroud, 1999]. Stancer et al. [1987, 1988], and Weitkamp and Stancer [1989] have reported excess sharing of haplotypes and non-random transmission of haplotypes in the HLA region in patients with BPAD and other affective disorders, although this finding has not been replicated [Goldin et al., 1982]. A recent study of lithium therapy and the HLA region has shown changes in HLA expression within 2 months of exposure to lithium [Kang et al., 2000], but in a small sample.

In light of the strong association results reported in schizophrenia and the possible overlap of genetic determinants with BPAD, we sought to investigate whether there is an association between NOTCH4 and BPAD in a sample of triads ascertained through a proband with BPAD. The two markers with the strongest evidence of association in the Wei and Hemmings [2000] study, along with three additional markers discovered in our laboratory, were studied. Both single-marker and 5-marker haplotype data were analyzed with family-based association methods. No genome-wide significant association was detected between any of the five SNP markers and BPAD in this sample. One marker showed nominal evidence of association ($P = 0.049$), but this evidence was not strengthened by haplotype analyses including nearby flanking markers or by case-control analysis using 93 Caucasian Centre d'Etude du Polymorphisme Humain (CEPH) controls. We conclude that the genetic markers we studied near NOTCH4 are not associated with BPAD in our sample.

MATERIALS AND METHODS

Study Subjects

Patient ascertainment was conducted by the NIMH Genetics Initiative Study [Nurnberger et al., 1997]. Briefly, ascertainment in the systematic group of families required a bipolar I (BPI) proband with a sibling affected by BPI or schizoaffective-bipolar disorder (SABP) and one or both parents unaffected by BPADI. The non-systematic ascertainment required two additional affected relatives with BPI, SABP, BPII disorder, or recurrent unipolar disorder (RUP). BPI, SABP, and RUP were defined by DSMIII-R criteria, BPII by RDC, but with the additional requirement of recurrent episodes of major depression. All subjects were assessed with the diagnostic instrument for genetic studies (DIGS) [Nurnberger et al., 1994]. Best-estimate diagnoses were assigned by two independent clinicians who reviewed the DIGS, family history, and any medical records. DNA samples of 153 BPAD probands, 147 affected sibling pairs, and 231 parents of BPAD probands were included in the present study.

A control group was procured from the Coriel Cell Repository, consisting of 93 unrelated founders from the CEPH (http://www.cephb.fr) Utah and French pedigree samples. Pedigrees with known mood disorders were excluded.

Markers

Two markers, designated as "SNP2" and "CTG microsatellite" by Wei and Hemmings [2000] were chosen due to their reported highly significant association with schizophrenia. SNP2, located in the promoter region of NOTCH4 is an A→G polymorphism. This marker was associated with schizophrenia at a P-value of 0.000036. The second marker, a CTG microsatellite in exon 1, was associated with schizophrenia at a P-value of 0.002 [Wei and Hemmings, 2000]. The haplotype consisting of SNP2 and the CTG microsatellite gave the most significant association result in the schizophrenia study, $P = 0.0000078$ [Wei and Hemmings, 2000]. The primer sequences for SNP2 and the CTG microsatellite were kindly provided by the authors [G. Hemmings, e-mail, August 2000].

Additional markers, flanking SNP2 and CTG, were developed in our laboratory by resequencing. These markers are hereafter referred to as SNP 3A, DISC SNP, and SNP 1341 (Fig. 1). SNP 3A is approximately 1.2 kb inside of the 5′ end of NOTCH4 and is a C→T polymorphism. DISC SNP is approximately 1 kb outside of the 5′ end of NOTCH4 and is a T→C polymorphism. SNP 1341 is approximately 1 kb outside of the 3′ end of NOTCH4 and is an A→G polymorphism.

The methodology for SNP discovery was as follows; a ~1 kb segment of DNA sequence was downloaded from the Golden Path browser (http://genome.ucsc.edu/) and imported into Oligo 6.4, which was used to design sequencing primers that met the following criteria: 18–21 bp, Tm 55–70°C, and no secondary structure. Selected primers were used to amplify each of 20 DNA samples from CEPH controls (individuals: 12399, 12400, 12385, 13133, 12466, 12455, 12716, 12717, 12708, 7016, 7050, 7057, 12571, 12572, 12560, 12556, 12557, 12548, 4477, 4479). The PCR protocol was 94°C for 12 min, followed by 15 cycles of 94°C for 45 sec, 66°C for 45 sec, then step down −1°C/cycle and 72°C for 90 sec. Then 30 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 90 sec. PCR product length verified on 2% agarose. The 60 μl of PCR product and 100 μl of ddH_2O were placed into each well of a 030 MANU 96-well plate. The plate was vacuumed at 22 psi for 10 min and 4 μl of DNA from each well was

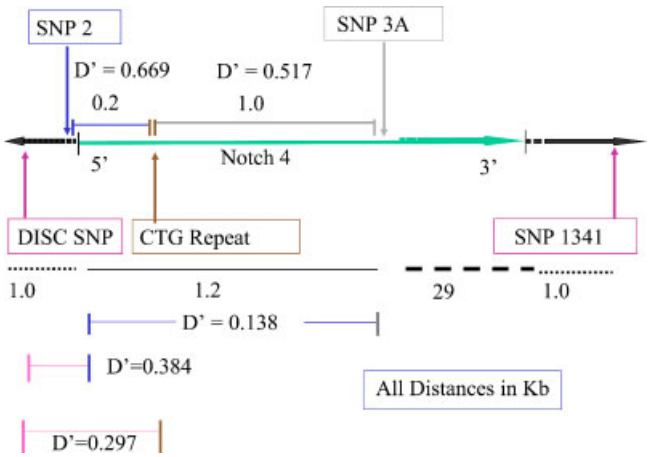

Fig. 1. Map of NOTCH4 markers used in this study. D' values and relative distances (kb) are shown for five SNPs genotyped in and near NOTCH4. For details, see Tables I–III.

then mixed with 4 μl Big Dye Terminator Mix (Applied Biosystems, Inc., Foster City, CA), 0.32 μl of 10 μM of sequencing primer, and 1.68 μl ddH_2O. This was denatured at 96°C for 2 min, then cycled 24 times through 96°C for 10 sec, 50°C for 8 sec, and 60°C for 4 min. Next, the wells of a MAHV 45 plate were filled with Sephadex (Sigma-Aldrich, Inc., St. Louis, MO) and primed with ddH_2O according to manufacturer's instructions. PCR products were then added to each Sephadex column, and centrifuged at 2,300 rpm for 10 min to extract purified product. This product was dry vacuumed for 30 min and loaded onto an ABI 3700 sequencer (Applied Biosystems, Inc.). Basepairs were called (Genotyper 3.6 by Applied Biosystems, Inc.) and DNA sequences were aligned and compared with Sequencher 4.1 (Gene Codes, Inc., Ann Arbor, MI).

Genotyping Methods

SNP2. SNP2 was located within a MspI restriction site. Genomic DNA was amplified by PCR, digested with MspI, and resolved on 2% agarose gels. PCR was performed with 25 ng of genomic DNA in a mixture containing 2.5 μl 10× PCR buffer (100 mM Tris-HCl, 35 mM $MgCl_2$, 250 mM KCl), 0.75 μl of 10 mM dNTP, 20.85 μl of distilled water, 1.6 μl of forward primer, 1.6 μl of reverse primer, and 0.2 μl of Taq DNA polymerase. Samples were denatured at 94°C for 12 min, followed by 15 cycles of 94°C for 45 sec, 66°C for 45 sec, then step down −1°C/cycle and 72°C for 90 sec. Then 30 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 90 sec. The 7.5 μl of PCR product was mixed with 0.2 μl MSP-1 restriction digest, 2 μl reaction 1 buffer, and 5.3 μl of water, then incubated at 37°C for 8 hr. Restriction digest was then electrophoresed on 2% agarose gel stained with ethidium bromide. Allele scoring was completed blindly by two independent readers.

CTG. CTG genotyping was performed on an ABI 3700 sequencer (Applied Biosystems, Inc.). PCR product was obtained using the same mixture and thermocycling specifications as SNP2. The forward primer was fluorescently labeled with FAM. The reverse primer incorporated added nucleotides GTTTCTT to the 5′ end to prevent "stuttering" of the genotyped product. Alleles were scored using Genotyper 3.6 (Applied Biosystems, Inc.) and verified manually. Three percent of genotypes failed to Mendelize and were dropped.

SNPs discovered in our laboratory. Genotyping for SNP 3A and 1341, and DISC SNP was performed using single base extension with fluorescence-polarization detection [Kwok, 2000]. The PCR mix was 1 μl 10× PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 0.5 μl 50 mM $MgCl_2$, 0.2 μl dNTP mix, 0.05 Platinum Taq DNA polymerase, and 7.25 μl distilled water run at the thermocycling specifications of SNP2. The 1 μl shrimp alkaline phosphatase (SAP), 1 μl SAP buffer, 0.1 μl exonuclease, and 7.9 μl distilled water were added to each PCR product well and incubated for 45 min at 37°C and then 95°C for 15 min. The 2.0 μl 5× TDI buffer, 0.05 μl thermosequenase (8 U/μl), 0.05 μl of four dye mix (equal parts Bodipy A, Rox G, Tamra C, R6G T), and 6.9 μl distilled water, 0.6 μl probe (10 μM) was added to each well and run with the following protocol: 93°C for 2 min, 50 cycles of 93°C for 10 sec, 50°C for 30 sec. Additional steps to enhance plate reading were necessary for SNP 3A. SSDNA bp 0.1 μl, 5× TDI buffer 2.0 μl, and distilled water 8.0 μl incubated at 37°C for 1 hr. Following this 10 μl 5× TDI buffer, 25 μl ethanol, and 40 μl distilled water were added to each well. These steps facilitated clearer plate reading. Data from the LJL Biosystems Analyst AD plate reader underwent cluster analysis to score genotypes at ≥99.9% confidence [Akula et al., 2002].

Statistical Methods

Hardy–Weinberg equilibrium. Agreement with expectations of Hardy–Weinberg equilibrium was tested using the programs HWSIM (biallelic markers) and MULT (multi-allelic markers). No significant deviations from Hardy–Weinberg equilibrium were observed.

Transmission disequilibrium test (TDT). The TDT [Spielman et al., 1993, as implemented by Genehunter 2.0] was used to test for association between each of the five individual SNPs and BPAD. In the absence of either linkage or association between marker and disease loci, marker alleles will be transmitted randomly from parents to offspring.

Haplotype association testing. To increase the proportions of informative triads for association mapping, we generated 3-marker haplotypes and tested them for association with bipolar disorder using TRANSMIT 2.5 [default settings; Clayton, 1999]. Although the methods used by TRANSMIT are not immune to population stratification [reviewed in Schulze and McMahon, 2002], TRANSMIT is a powerful method for detecting association when data from multiple linked markers are available. TRANSMIT tests for association between genetic markers and disease by examining the transmission of marker haplotypes from parents to affected offspring, comparing the observed transmission frequencies with those expected under the null hypothesis of no association. TRANSMIT can deal with transmission of multi-locus haplotypes, even if phase is unknown, and some parental genotypes may be unknown. Clayton [1999] addresses the theory underlying the method.

Linkage disequilibrium (LD). Pairwise LD between all markers was calculated with GOLD (www.well.ox.ac.uk/asthma/GOLD; see Table III). GOLD uses founder haplotypes identified by SIMWALK2 to calculate the absolute value of the multiallelic version of the standardized disequilibrium coefficient D' [Lewontin, 1964, 1988] and other measures of LD. The calculation of LD by GOLD also incorporates a standard contingency table chi-squared test for intermarker association [Abecasis and Cookson, 2000] (Fig. 2).

Transmission distortion. To test for possible transmission distortion that might lead to a spurious association finding in the TDT, we also genotyped SNP2 in a control group of 93 unrelated CEPH founders. Genotype and allele frequencies were compared by the chi-squared test.

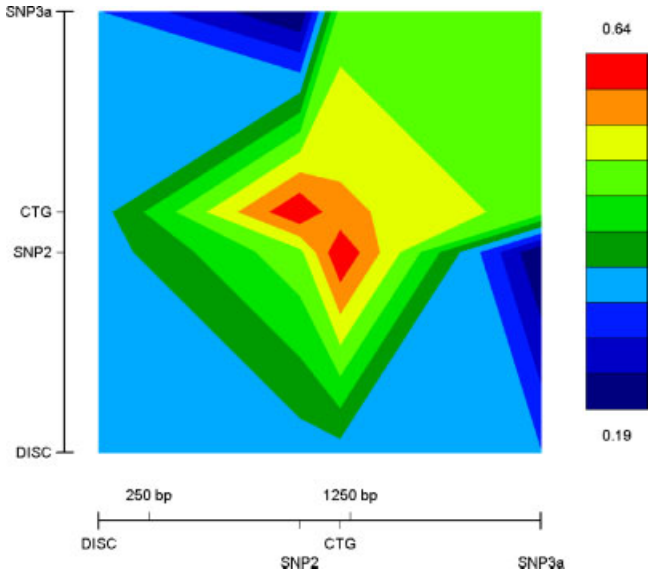

Fig. 2. Intermarker linkage disequilibrium (LD) between studied NOTCH4 markers. The Gold program plots the markers and their relative respective distances along with D' values. The orange at the top of the color graph scale depicts high D' values while the dark blue at the bottom depicts low D' values. The overall figure mirrors itself across the diagonal. There is strong LD between SNP2 and the CTG microsatellite with a gradual reduction of LD elsewhere.

RESULTS

Single Marker Analyses

No genome-wide significant results were obtained for any of the five markers tested (Table I). SNP2, which was informative in 75 triads, showed nominal evidence of association ($\chi^2 = 3.85$, $P = 0.049$). Allele 4 of the CTG microsatellite, located 0.2 kb from SNP2, revealed a similar transmission/non-transmission ratio as SNP2, but this was not significant in the small number of triads informative for this marker in our sample.

TABLE I. TDT Results for the Five Genotyped Markers*

	T	UT	χ^2	*P*-value
DISC SNP				
Allele 1	26	27	0.02	0.89
Allele 2	27	26		
SNP2				
Allele 1	46	29	3.85	0.049+
Allele 2	29	46		
CTG microsatellite				
Allele 1	1	0	1.00	0.32
Allele 2	20	16	0.44	0.51
Allele 3	37	25	2.32	0.13
Allele 4	28	43	3.17	0.08
Allele 5	18	15	0.27	0.60
Allele 6	13	18	0.81	0.37
Allele 7	3	3	0.00	1.00
SNP 3A				
Allele 1	16	24	1.60	0.21
Allele 2	24	16		
SNP 1341				
Allele 1	36	28	1.00	0.32
Allele 2	28	36		

*Nominal *P*-values <0.05 are indicated with a (+). T, transmitted; UT, untransmitted.

Intermarker LD

Pairwise LD was estimated between all possible marker pairs (Table III). There was highly significant, but imperfect, LD between all marker pairs except SNP2 and SNP 1341.

Haplotype Analyses

To clarify any possible association between SNP2 with BPAD, we constructed 3-marker haplotypes containing SNP2 (Table II). In addition to SNP2, the only marker that showed any evidence of association in single-marker analysis, two markers that flank SNP2 were chosen, DISC SNP and the CTG microsatellite. Without correction for multiple testing, the haplotypes 1-2-4 and 1-1-3 showed some evidence of unequal transmission in individual haplotype tests. This follows the expected trend of SNP2 and the CTG microsatellite transmitting unequally. Global results were not significant.

Transmission Distortion

SNP2 genotype and allele frequencies were determined in a set of 93 unrelated controls. Chi-square analyses indicated no significant difference in allele frequency ($P = 0.512$) or genotype frequency ($P = 0.406$) between controls and BPAD probands (see Table IV).

DISCUSSION

This study tested the hypothesis that markers near NOTCH4 were associated with BPAD. We tested two markers previously associated with schizophrenia [Wei and Hemmings, 2000] and three markers discovered in our own laboratory in a sample of triads with good power to detect an association, if one existed. A marginal association was found with one marker, SNP2. Both single-marker and 3-marker haplotype tests were performed. Haplotype analysis failed to strengthen this association. Further testing in a case-control analysis undermined any evidence of association. On balance, either the marginal association by TDT is spurious or due to possible transmission distortion. No consistent evidence of association was observed. We conclude that there is no evidence that the NOTCH4 markers we studied are associated with bipolar disorder in this sample.

Our study is limited in several ways. We did not attempt to uncover all variation within the NOTCH4 region we studied, so it is possible that genetic variation associated with bipolar disorder still exists in this region. This is unlikely, however, since most of the adjacent SNPs we studied were in LD with each other and thus should have revealed association with a putative disease locus lying between them.

These results suggest that *NOTCH4* is not an important susceptibility gene for bipolar disorder in this sample. The nominally-significant over-transmission we observed at one marker was most likely the result

TABLE II. Association Results for Haplotypes Generated for the Markers DISC SNP, SNP2, and CTG*

Haplotype	Observed (O)	Expected (E)	Variance (O−E)	χ^2	*P*-value (1 df)
2.1.1	1.00	0.57	0.25	0.75	0.386
2.2.1	1.02	0.58	0.25	0.78	0.377
1.1.2	1.63	2.44	0.71	0.91	0.340
2.1.2	25.06	23.89	9.10	0.15	0.699
1.2.2	1.07	1.76	0.73	0.64	0.424
2.2.2	1.04	0.59	0.25	0.8	0.371
1.1.3	58.87	46.98	15.40	9.17	0.002+
2.1.3	14.06	13.81	4.74	0.01	0.920
1.2.3	18.19	17.41	6.28	0.1	0.752
2.2.3	0.10	1.20	0.50	2.44	0.118
1.1.4	5.29	4.88	1.95	0.09	0.764
1.2.4	80.49	94.14	21.02	8.86	0.003+
2.2.4	22.72	20.31	7.26	0.8	0.371
2.1.5	3.52	2.87	0.98	0.43	0.512
1.2.5	31.96	29.38	10.18	0.66	0.417
2.2.5	1.02	0.68	0.23	0.5	0.480
1.2.6	26.81	32.12	10.38	2.7	0.100
1.1.7	1.03	0.61	0.24	0.7	0.403
1.2.7	4.08	5.16	1.68	0.7	0.403
2.2.7	1.02	0.60	0.24	0.74	0.390

*Global chi-square (df = 19) = 25.15, $P = 0.12$. Nominal *P*-values <0.05 are indicated with a (+).

TABLE III. Pairwise D' Values for all Possible Marker Pairs*

Marker 1	Marker 2	N	D'	df	χ^2	*P*-value
DISC SNP	SNP2	509	0.384	1	45.46	0.000
DISC SNP	CTG	511	0.297	5	90.37	0.000
DISC SNP	SNP 3A	459	0.269	1	5.07	0.024
DISC SNP	SNP 1341	512	0.393	1	5.23	0.022
SNP2	CTG	513	0.669	5	220.01	0.000
SNP2	SNP 3A	459	0.138	1	7.54	0.006
SNP2	SNP 1341	512	0.055	1	0.76	0.384
CTG	SNP 3A	462	0.517	5	124.82	0.000
CTG	SNP 1341	519	0.505	5	162.64	0.000
SNP 3A	SNP 1341	461	0.513	1	50.32	0.000

*N, number of individuals with complete data for comparison.

of random fluctuation, since it was not strengthened by haplotype tests and was not confirmed in the case-control experiments. It is also possible that this apparent over-transmission represents transmission distortion, which can create an appearance of significant over-transmission of marker alleles that will not be supported by case-control tests. Since tests of transmission distortion were not reported by Wei and Hemmings [2000], we cannot rule out a role for this phenomenon in that study.

TABLE IV. Results of Case-Control Association Testing of SNP2 in Probands and Controls

Genotype	Probands	Frequency	Controls	Frequency
Genotype-wise comparison ($\chi^2 = 1.804$, df = 2, $P = 0.406$)				
PP	61	0.41	45	0.48
PQ	66	0.44	38	0.41
QQ	23	0.15	10	0.11
Allele-wise comparison ($\chi^2 = 0.429$, df = 1, $P = 0.512$)				
Allele				
P	188		128	
Q	112		86	

We found haplotype-based tests in prior studies to be an effective approach to association studies. However, uncertainties remain as to the ideal way to reconstruct haplotypes in small family structures [Schaid et al., 2002]. Finally the optimal set of markers to use for haplotype reconstruction is also not clear, but emerging data regarding the existence of haplotype blocks within the human genome may help guide future studies [Taillon-Miller et al., 2000]. Further study is needed in this area.

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