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Modest Evidence for Linkage and Possible Confirmation of Association Between NOTCH4 and Schizophrenia in a Large Veterans Affairs Cooperative Study Sample

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Wei and Hemmings [2000: Nat Genet 25:376–377], using 80 British parent–offspring trios, identified a number of NOTCH4 variants and haplotypes that showed statistically significant evidence of association to schizophrenia. Specifically, the 10 repeat allele of a $(CTG)_n$ marker and the 8 repeat allele of a $(TAA)_n$ marker demonstrated excess transmission to affected individuals; SNP2[1] and haplotypes SNP2-$(CTG)_n$ and SNP1[2]-SNP2-$(CTG)_n$ also showed significant associations. In an attempt to replicate these findings, we tested for linkage and association between the same five markers used by Wei and Hemmings in 166 families collected from a multi-center study conducted by the Department of Veterans Affairs (DVA) Cooperative Study Program (CSP). The families include 392 affected subjects (schizophrenia or schizoaffective disorder, depressed) and 216 affected sibling pairs. The families represent a mix of European Americans (n = 62, 37%), African Americans (n = 60, 36%), and racially mixed or other races (n = 44, 27%). We identified moderate evidence for linkage in the pooled race sample (LOD = 1.25) and found excess transmission of the 8 ($P = 0.06$) and 13 ($P = 0.04$) repeat alleles of the $(TAA)_n$ marker to African American schizophrenic subjects. The 8 and 13 repeat alleles were previously identified to be positively associated with schizophrenia by Wei and Hemmings [2000: Nat Genet 25:376–377] and Sklar et al. [2001: Nat Genet 28:126–128], respectively.

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[1]SNP2 is a T→C substitution resulting in an Msp1 site.
[2]SNP1 is an A→G substitution resulting in an Msp1 site.

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INTRODUCTION

A number of positive linkage findings have been reported on chromosome 6p, beginning with the identification by Straub et al. [1995] and Wang et al. [1995] of linkage to schizophrenia at 6p22-6p24. Many studies, based on samples of various ethnicities and using different diagnostic methods, have replicated these findings [Antonarakis et al., 1995; Moises et al., 1995; Schwab et al., 1995; Schizophrenia Linkage Collaborative Group (SLCG), 1996; Brzustowicz et al., 1997; Lindholm et al., 1999; Hwu et al., 2000]. Several groups have also reported linkage between schizophrenia and chromosome 6q [Cao et al., 1997; Kaufmann et al., 1998; Martinez et al., 1999; Schwab et al., 2000]. Nurnberger et al. [1999] and Daniels et al. [1997] offer helpful reviews of these earlier linkage findings. Nurnberger et al. [1999] also discusses some of the candidate genes within these regions that have been targets of association studies. Turecki et al. [1997] performed a meta-analysis of nine previous studies that reported linkage on chromosome 6p. Unlike the majority of the studies above, which showed linkage peaks between 6p22 and 6p24, they found the strongest evidence for linkage at 6p21.3. Hwu et al. [2000], Schwab et al. [2000], and McGinnis et al. [2001] have since confirmed linkage in this region. The highest number of association studies have focused on this 6p21.3 region.

Several studies encouraged by the consistent positive linkage results on chromosome 6p, sought to test for association between markers in candidate gene regions and schizophrenia. Wei and Hemmings [2000], using 80 British parent–offspring trios, scanned this region using the transmission disequilibrium test (TDT) [Spielman et al., 1994] on 13 markers within the MHC region (6p21.3). They found a marker in locus HSMHC3A5, known to contain the NOTCH4 gene, to be associated with schizophrenia. They proceeded to genotype four additional markers within NOTCH4: three in the 5′ flanking region, one in exon 1, and another in intron 17, and conducted TDT tests on each marker as well as on several two and three marker haplotypes. The positions of these markers are shown in Figure 1. Wei and Hemmings [2000] found significant association results for markers $(TAA)_n$ and $(CTG)_n$ ($P = 0.00017$ and $P = 0.000036$, respectively), while the marker haplotypes with the most significant associations were SNP2-$(CTG)_n$ and SNP1-SNP2-$(CTG)_n$ ($P = 0.0000078$ and $P = 0.000011$, respectively). An excess of the 10 copy allele of the $(CTG)_n$ marker, $(CTG)_{10}$, and the 8 copy allele of the TAA marker, $(TAA)_8$, were found to be transmitted preferentially to schizophrenic offspring.

Sklar et al. [2000] tested all of the positive markers used by Wei and Hemmings [2000] for association using German Israeli, German, and Bulgarian family based samples, and a British case-control sample. They confirmed one previous finding of linkage to the NOTCH4 area in the German Israeli sib-pairs. Also, as reported in their Table I, but not noted in their text, they found that the 13 repeat allele of the $(TAA)_n$ marker demonstrated excessive transmission to schizophrenics in the German Israeli sample ($P = 0.018$) and in their combined sample ($P = 0.032$). McGinnis et al. [2001], using 300 Scottish schizophrenics and 600 controls, tested for association using markers $(CTG)_n$ and $(TAA)_n$, but found no strong evidence for association ($P = 0.15$,

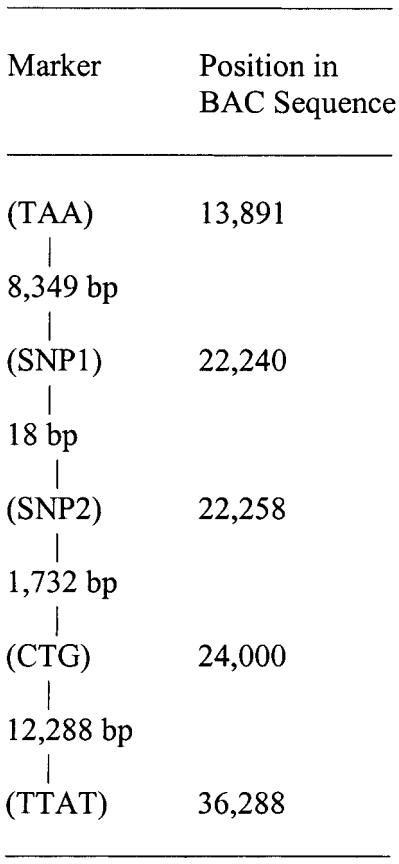

Fig. 1. Position of NOTCH4 markers on chromosome 6p21.3, genomic clone U89335. (Genbank [http:www.ncbi.nlm.nih.gov] GI: 1841541.) Genetic distances between markers indicated by the number of base pairs.

TABLE I. Number of Families and Affected Individuals Included in Each Analysis

	Narrow		Broad	
Race	Number of families	Number affected	Number of families	Number affected
AA	44	101	69	161
EA	52	109	64	142

AA, African America; EA, European American. For the narrow ethnic group, both parents are of the same ethnic group (both either AA or EA). For the broad ethnic group categories, families with either one AA parent or two-mixed race parents where each parent was part AA were added to the AA group; while families that had at least one EA parent and the second parent who was non-AA were added to the EA group.

$P = 0.16$). Two Japanese case-control studies have also been performed, one using SNP1, SNP2, and $(CTG)_n$ and the other using only $(CTG)_n$; neither found evidence of association ($P = 0.13$ [at $(CTG)_n$] and $P = 0.71$, respectively) [Imai et al., 2001; Ujike et al., 2001]. Most recently, Fan et al. [2002] examined all five markers used by Wei and Hemmings [2000] using a Chinese sample of 544 cases and 621 controls and 300 trios. Their most significant result was from testing for different allele frequency distributions between cases and controls at the $(TTAT)_n$ marker ($P = 0.03$). The TDT for this marker yielded a P-value of 0.13. Swift-Scanlan et al. [2002] examined the $(CTG)_n$ marker in 65 multiplex bipolar pedigrees, postulating that there are shared genes involved in both schizophrenia and bipolar disorder. They found modest association between the $(CTG)_{13}$ allele and schizophrenia ($P = 0.01$). However, their result became less significant when they restricted their sample to a subset of 30 families that contained family members that exhibited psychotic symptoms ($P = 0.10$).

In this study, we assess evidence for linkage and association between schizophrenia and the five NOTCH4 markers used by Wei and Hemmings [2000] in 166 families collected through the Department of Veterans Affairs (DVA) Cooperative Study Program (CSP) [Tsuang et al., 2000]. We find suggestive evidence for linkage to schizophrenia to this region and, intriguingly, excess transmission of both $(TAA)_8$ and $(TAA)_{13}$ alleles to schizophrenics in our African American families—these are the same two alleles identified by Wei and Hemmings [2000] and Sklar et al. [2001], respectively. Unfortunately, this finding was not replicated in our European American families. Additionally, we were not able to replicate Wei and Hemmings [2000] positive association to $(CTG)_n$, nor find association to any of the other three markers used in their study.

MATERIALS AND METHODS

The DVA CSP collected clinical data and blood samples from 166 families ascertained through a pair of schizophrenic siblings. A comprehensive description of the sample and methods of data collection has been published [Tsuang et al., 2000]. Family members were classified as affected if they had a diagnosis of schizophrenia or schizoaffective, depressed, based on the Diagnostic Interview for Genetic Studies (DIGS) [Nurnberger et al., 1994; Faraone et al., 1996]. This is the same classification scheme used by Wei and Hemmings [2000].

DSM-III-R diagnoses were made by two senior psychiatrists or psychologist investigators with clinical and research experience in the diagnosis of psychosis. The diagnosis was based on the content of the DIGS interview, information provided by relatives who had been interviewed with the Family Interview for Genetic Studies (FIGS), and a review of medical records [Tsuang et al., 2000, 2001; Faraone et al., 2002].

Among the 166 families, 124, 30, 7, 4, and 1 had two, three, four, five, and six affected members, respectively. There are a total of 216 affected sibling pairs in these families. The mean number of individuals sampled per family was 4.7. Most of the affected subjects ($n = 361$; 92.1%) met DSM-III-R criteria for schizophrenia. The remainder ($n = 31$) met criteria for schizoaffective disorder, depressed. A detailed description of the linkage analysis sample has been previously reported.

Genotypes were determined by PCR amplification of polymorphic loci using primers labeled with fluorescent probes [Tsuang et al., 2001]. PedCheck was used to identify genotypes incompatible with Mendelian inheritance [O'Connell and Weeks, 1998].

Linkage and Linkage Disequilibrium Analysis

We performed semi-parametric linkage analysis using the exponential model of Genehunter Plus [Kong and Cox, 1997], a modified version of Genehunter [Kruglyak et al., 1996], to confirm linkage to the NOTCH4 region in our sample. We used the S_{pairs} score that assesses identity by descent (IBD) sharing among all pairs of affected individuals within families.

We used two approaches to assess evidence of linkage disequilibrium between schizophrenia and any of the five NOTCH4 markers. The first is the pedigree disequilibrium test (PDT) which takes pedigrees and breaks them into mother–father–affected offspring trios and discordant sib pairs (DSP) [Martin et al., 2000]. For the trios, it calculates the difference between the number of transmissions and non-transmissions of an allele to the affected offspring. For DSP, the difference between allele frequency in the affected and unaffected sibs is calculated. For each pedigree, these two values are combined; all pedigree values are then summed and standardized, yielding a statistic that is asymptotically normally distributed and has power to detect deviations from the Mendelian transmission rates [Martin et al., 2001].

The second approach uses the family based association test (FBAT) [Rabinowitz and Laird, 2000]. In addition to being able to use extended families, the FBAT also handles missing data appropriately, and so does not waste information. Association between a specific allele and schizophrenia is assessed using a test statistic that sums a function of the number of alleles possessed by each affected individual over all individuals. The function of the number of alleles depends on the genetic model being considered. An empirical estimate of the variance is used, as recommended by Xu et al. [2001], to account for the fact that we are looking for association in the presence of linkage. Evidence for association to a given marker is determined by appropriately combining the allele specific test statistics.

In addition to the marker-disease associations, we also tested the four most significant haplotype-disease associations found by Wei and Hemmings [2000], namely $(TAA)_n$-SNP1, SNP2-$(CTG)_n$, $(TAA)_n$-SNP1-SNP2, and SNP1-SNP2-$(CTG)_n$ using both FBAT and PDT. We used Merlin [Abecasis et al., 2002] and Fugue [Abecasis et al., 2001] to infer the most likely haplotypes for each family.

Before performing the linkage or association analyses, we divided the families into racial groups based

on the probands' parents' races as identified in the DIGS. For the narrow definition we included only those 122 families in which both parents are of the same racial group (both either African American, n = 60, or European American, n = 62). For the broad racial group categories, 31 families were added to the African American group. These families had either one African American parent or two-mixed race parents where each parent was part African American. Thirteen families were added to the European American group. These families had at least one European American parent and the second parent was non-African American.

We used 75 previously genotyped markers from four chromosomes to look for evidence of mispecified familial relationships. Of the 166 families, 30 had repeated genotype incompatibilities at many markers. For 14 families, reported pedigree structure was incorrect but could easily be corrected by use of the available genotype information [Boehnke and Cox, 1997]. Correct relationships for the remaining 16 families could not be resolved with our present data, and those families were excluded from the current analysis. We identified a single family with a genotyping error and excluded the data from the marker with the error for the entire family. Finally, seventeen additional families were excluded because they were missing enough genotype data that they provided no linkage information. Table I lists the races and number of families and affected individuals included in each analysis after exclusions.

We performed the linkage and PDT association tests on each racial group independently and again with all pedigrees combined. Since few observations per allele were available when running FBAT on a single race, we only obtained results for this test for all pedigrees combined.

RESULTS

Table II shows a summary of the linkage results using both definitions of race. We found suggestive evidence for linkage to the NOTCH4 region when using all families and the broad definition of race (LOD = 1.25) and weaker evidence using the narrow definition (LOD = 0.58). When considering African Americans alone, LOD scores of 0.81 and 0.65 were attained for the broad and narrow definitions of race, respectively. European Americans alone had LOD scores of 0.45 and 0.08.

The results of a global test for association for each marker and haplotype using the PDT are summarized in Table III. None of the markers for either of the races reached statistical significance after correcting for multiple tests, although $(TAA)_n$ was nominally significant at the 0.05 level for African Americans. The 8 and 13 repeat alleles of this marker were found more often in schizophrenic sibs than unaffected siblings ($P = 0.06$, $P = 0.04$), while the 12 repeat allele was more frequently found in unaffected siblings ($P = 0.03$). Interestingly, the 8 repeat allele was found to be the most significant excessively transmitted allele by Wei and Hemmings [2000] ($P = 0.0002$), while Sklar et al. [2001] found the 13 copy allele to be the allele most excessively transmitted to affecteds ($P = 0.032$). These transmission differences were not observed in the European Americans in our sample. Tables IV and V show the allele frequency distribution and the number of transmissions and nontransmissions of alleles from heterozygous parents to affected offspring for our EA, AA, and combined sample, for Wei and Hemmings [2000] sample and for the Sklar et al. [2001] sample.

Table VI summarizes the results of the tests for association using the FBAT. The most significant result was in marker $(TTAT)_n$ using the recessive model. The multi-allelic test for the marker produced a *P*-value of 0.10, which was driven by an excess transmission of the most common allele to affected individuals ($P = 0.05$).

Although the association result for $(TAA)_n$ was significant at the 0.05 level, it does not remain significant after correcting for the multiple tests performed. An appropriate significance level is difficult to determine since most of the tests that were performed are dependent and so a Bonferonni adjustment would be overly conservative. However, given that well over 100 tests were performed and that tests at each marker are close to being independent, it is likely that the appropriate significance threshold is at least an order of magnitude less than 0.05. It is, however, also appropriate to note that we are conducting a follow-up investigation and that the $(TAA)_n$ alleles that we found to be most significantly associated with schizophrenia are the same as those previously identified by Wei and Hemmings [2000] and Sklar et al. [2001]. The interpretation is further complicated since our positive results do not extend to our European American sample.

TABLE II. Linkage Results

Race definition	Narrow	Broad
AA	0.65	0.81
EA	0.08	0.45
Combined	0.58	1.25

Maximum LOD scores using narrow and broad race classifications as specified in Table I.

TABLE III. Global Test for Associations for Each Marker and Haplotype Using the Pedigree Disequilibrium Test (PDT), the Lowest *P*-Value is Reported (not Corrected for Multiple Comparisons)

Race	AA	EA	Combined
$(TAA)_n$	0.04	0.38	0.29
SNP1	0.49	0.82	0.51
SNP2	0.22	0.43	0.89
$(CTG)_n$	0.76	0.23	0.93
$(TTAT)_n$	0.32	0.64	0.27
$(TAA)_n$-SNP1	0.57	0.41	0.88
$(TAA)_n$-SNP1-SNP2	0.32	0.60	0.72
SNP1-SNP2-$(CTG)_n$	0.19	0.68	0.38
SNP2-$(CTG)_n$	0.18	0.78	0.33

P-values are based on the sumPDT statistic.

TABLE IV. Allele Transmission in Trios and Discordant Sib Pairs (DSP) for Marker $(TAA)_n$

Allele		VA combined	VA AA	VA EA	Wei and Hemmings	Sklar et al.
7	T:N	0:2	0:2	0:0	1:1	17:8
	Aff:Unaff	5:7	2:3	3:4		
8	T:N	20:26	6:2	14:14	53:21	152:150
	Aff:Unaff	90:77	11:5	79:72		
9	T:N	27:27	19:17	8:10	22:35	139:139
	Aff:Unaff	87:78	35:33	52:43		
10	T:N	8:6	4:4	4:2	22:32	120:139
	Aff:Unaff	68:52	28:20	8:7		
11	T:N	2:7	0:6	2:1	5:7	11:15
	Aff:Unaff	19:21	11:14	8:7		
12	T:N	2:4	0:2	2:2	7:13	19:27
	Aff:Unaff	19:17	11:12	8:5		
13	T:N	4:1	2:0	2:1	5:4	32:17
	Aff:Unaff	28:19	21:11	7:8		
14	T:N	5:5	5:3	0:2	4:6	41:32
	Aff:Unaff	12:15	7:6	5:9		
15	T:N	0:0	0:0	0:0	0:0	2:5
	Aff:Unaff	0:0	0:0	0:0		

T:N are the numbers of transmissions and non-transmissions of the allele from heterozygous parent to affected offspring, respectively. Aff:Unaff are the numbers of alleles possessed by affected and unaffected members of a discordant sib pair, respectively.

We repeated our association analysis again using a more restrictive definition of schizophrenia by reassigning 21 subjects diagnosed as schizoaffective disorder, depressed (SZD) (11 AA and 10 EA) to the unaffected group. The results from linkage analysis and tests of association using FBAT changed only nominally. Marker $(TAA)_n$ was found to have the strongest association to schizophrenia when using FBAT and the narrower definition of schizophrenia ($P = 0.14$). The results from the PDT also changed very little. Recall that when using the broader definition of schizophrenia, association to $(TAA)_n$ marker was found to be nominally significant in the AA sample ($P = 0.04$). Although the strength of this association decreased slightly ($P = 0.06$), the evidence for association between the 8 repeat allele and schizophrenia weakened substantially ($P = 0.27$ [SZD excluded], $P = 0.06$ [SZD included]). The evidence for association to the 13 repeat allele did not change significantly. The largest increase in evidence for association was observed for marker $(CTG)_n$ ($P = 0.08$ [SZD excluded], $P = 0.23$ [SZD included]). The significance at this marker is driven by the $(CTG)_{11}$ allele being transmitted to affected individuals twice as often as to unaffected ($P = 0.02$). An association with this allele was previously reported in the German Israeli sib pair sample of Sklar et al. [2001] ($P = 0.003$) and by Swift-Scanlan et al. [2002] using a sample of subjects with psychotic symptoms and broadly defined bipolar illness ($P = 0.05$). However, in both of these samples this allele was transmitted more often to the unaffected sibs.

DISCUSSION

Using our Veteran's Affair Cooperative Study sample [Tsuang et al., 2000], we found moderate evidence for linkage of schizophrenia to the NOTCH4 region using the combined race sample, a broad definition of race and an affection status including schizoaffective, depressed, and schizophrenia (LOD = 1.25). This result is consistent with a number of previous studies [Turecki et al., 1997; Hwu et al., 2000; Schwab et al., 2000; McGinnis et al., 2001]. Our most significant association result was for marker $(TAA)_n$ ($P = 0.04$), which, due to the large number of tests performed and previously significant findings by Wei and Hemmings [2000] and Sklar et al. must be interpreted with caution. The two most significant alleles in the African American families were $(TAA)_8$ and $(TAA)_{13}$ which were transmitted more frequently to affected than unaffected individuals ($P = 0.06$, $P = 0.04$). The excessive transmission of the 8 repeat allele is consistent with Wei and Hemmings [2000] results and the excess transmission of the 13 copy allele was also found by Sklar et al. [2001]. However, these results were not replicated in our European American sample, which is more racially similar to both the Sklar et al. [2001] and Wei and Hemmings [2000] samples. It is unclear how to reconcile this discrepancy, although racial heterogeneity, gene–environment interaction, and/or admixture may contribute to the difference. An unknown epistatic locus may exist in the AA sample that modifies the effect of the 8 and 13

TABLE V. Allele Frequency Distribution in Samples Showing Evidence of Association

Sample/allele	7	8	9	10	11	12	13	14	15
VA (EA)	0.028	0.400	0.263	0.172	0.014	0.042	0.039	0.035	0.007
VA (AA)	0.063	0.138	0.188	0.193	0.084	0.079	0.111	0.128	0.017
VA (combined)	0.044	0.290	0.229	0.181	0.044	0.058	0.067	0.077	0.010
Wei and Hemming	0.084	0.311	0.240	0.227	0.050	0.084	0.038	0.042	0.000
Sklar et al.	0.017	0.364	0.287	0.176	0.002	0.039	0.043	0.067	0.006

TABLE VI. Tests for Association Using the Family Based Association Test, the *P*-Value for Each Marker and Haplotype is Reported

Model	Additive	Recessive	Dominant
$(TAA)_n$	0.21	0.89	0.34
SNP1	0.29	0.57	0.57
SNP2	0.67	0.73	0.73
$(CTG)_n$	0.85	0.56	0.93
$(TTAT)_n$	0.40	0.10	0.47
$(TAA)_n$-SNP1	0.47	0.65	0.56
$(TAA)_n$-SNP1-SNP2	0.46	0.73	0.58
SNP1-SNP2-$(CTG)_n$	0.21	[a]	0.35
SNP2-$(CTG)_n$	0.40	[a]	0.45

[a]Too few observations of recessive genotypes to perform calculation.
A minimum of ten informative families was necessary to compute the test statistic. The empirical variance was used to calculate the test statistic (recommended by Lake et al., 2001 when testing in an area of linkage).

repeat alleles, increasing the prevalence of schizophrenia in individuals inheriting these alleles. Ethnic admixture in our EA sample may have influenced our ability to detect association in that sample. Unfortunately, we do not have sufficiently detailed racial information to determine which EA individuals are of British descent.

We also tested for association between schizophrenia and the four haplotypes Wei and Hemmings [2000] found to be most strongly association with schizophrenia. We found no association between these haplotypes and schizophrenia. Although haplotypes were not able to be determined unambiguously, the most likely haplotypes were able to be inferred using Merlin and Fugue. The use of inferred haplotypes in our analysis could have adversely affected our ability to find an association, however, this is not likely given the small number and density of markers used.

We repeated the association and linkage analyses by restricting the assignment of schizophrenia only to those individuals diagnosed with schizophrenia. The significance of the association between $(TAA)_8$ and $(TAA)_{13}$ and schizophrenia decreased from *P*-values of 0.06 and 0.04 to 0.23 and 0.06, respectively. Aside from these changes and the increase in the evidence for association between the marker $(CTG)_n$ and allele $(CTG)_{11}$ and schizophrenia in the our EA sample, no other associations or linkage changed significantly.

Six previous studies have attempted to replicate Wei and Hemmings [2000] finding, two of which had samples that were ethnically similar to the original British sample. Neither McGinnis et al. [2001] Scottish sample nor Sklar et al. [2001] British case-control sample demonstrated significant association between NOTCH4 markers and schizophrenia. Both of these studies had sufficient sample sizes to detect gene effects of the magnitude suggested by Wei and Hemmings [2000] findings. Sklar et al. [2001] were also unable to find evidence for association using German and Bulgarian trios or German Israeli sib pairs.

Several possibilities could be considered to explain the discrepancy between Wei and Hemmings [2000] results and those of previous studies and our own. First, the variation in the assessment and or definition of the affected phenotype might explain the differences in the significance of association. Second, as suggested by McGinnis et al. [2001], it is possible that Wei and Hemmings [2000] sample contains a polymorphism in a susceptibility gene that is significantly more common in the Welsh. Third, it is possible that Wei and Hemmings [2000] positive findings, although highly significant, represent a false positive.

We recognize that our sample is less than ideal for an association study. We have only 21 families with fully genotyped trios, and although we have a total of 292 DSP, the low penetrance of schizophrenia results in overmatching of affected and unaffected siblings. These characteristics of our families and schizophrenia may have limited our power to detect associations [Boehnke and Langefeld, 1998]. Additionally, racial heterogeneity is present, so even though linkage was strongest using the combined race sample, susceptibility variants may be different, or at different frequencies in the two races. Hence, our failure to detect an association in the European American sample may represent a false negative.

In conclusion, our study presents intriguing evidence that an association may exist between schizophrenia and the $(TAA)_n$ locus of NOTCH4. However, it remains possible that other known genes in this region, such as PBX2, AGPAT1, AGER, and TNXA, are responsible for the suggestive linkage results. Associations between these genes and schizophrenia should be explored in future studies.

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