Association evidence of schizophrenia with distal genomic region of NOTCH4 in Taiwanese families

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Evidence for association with schizophrenia has been reported for NOTCH4, although results have been inconsistent. Previous studies have focused on polymorphisms in the 5′ promoter region and first exon of NOTCH4. Our aim was to test the association of the entire genomic region of NOTCH4 in 218 families with at least two siblings affected by schizophrenia in Taiwan. We genotyped seven single nucleotide polymorphisms (SNPs) of this gene, with average intermarker distances of 5.3 kb. Intermarker linkage disequilibrium (LD) was calculated using GOLD software, and single-locus and haplotype association analyses were performed using TRANSMIT software. We found that the T allele of SNP rs2071285 ($P = 0.035$) and the G allele of SNP rs204993 ($P = 0.0097$) were significantly preferentially transmitted to the affected individuals in the single-locus association analysis. The two SNPs were in high LD ($D' > 0.8$). Trend for overtransmission was shown for the T-G haplotype of the two SNPs to affected individuals ($P = 0.053$), with the A-A haplotype significantly undertransmitted ($P = 0.034$). The associated region distributed across the distal portion of the NOTCH4 gene and overlapped with the genomic region of the G-protein signaling modulator 3 and pre-B-cell leukemia transcription factor 2. In summary, we found modest association evidence between schizophrenia and the distal genomic region of NOTCH4 in this Taiwanese family sample. Further replication for association with the distal genomic region of NOTCH4 is warranted.

Keywords: Chromosome 6p, family-based association study, NOTCH4, schizophrenia

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Schizophrenia is a serious neuropsychiatric illness affecting 1% of the general population. Family, twin and adoption studies have shown that schizophrenia is predominantly genetically determined and has high heritability (McGuffin *et al.* 2003). The mode of transmission is still not clear, although a multilocus model is favored in which several genes, each having a small effect and acting in epistasis, lead to schizophrenia (Risch 1990). A number of positive linkage findings to schizophrenia have been reported on chromosome 6p (Antonarakis *et al.* 1995; Moises *et al.* 1995; Straub *et al.* 1995; Wang *et al.* 1995). Further, suggestive linkage evidence for chromosome 6p was reported in our earlier report using Taiwanese family sample (Hwu *et al.* 2000).

Wei and Hemmings (2000) studied the association of schizophrenia with four markers [single nucleotide polymorphism (SNP) 1, SNP2, (TAA)n, and (CTG)n] in the 5′ promoter region and first exon and one marker (TTAT)n in the intron 17 of NOTCH4 gene. They found highly significant association results with schizophrenia for markers (TAA)n, (CTG)n and SNP2 in 80 British parent–offspring trios. Follow-up studies focused on the four markers in the 5′ promoter region and first exon of this gene and the results were inconsistent. For example, in Caucasian samples, six studies observed no association of schizophrenia with some of the markers (Anttila *et al.* 2003; Carmine *et al.* 2003; Luo *et al.* 2004; McGinnis *et al.* 2001; Sklar *et al.* 2001; Wassink *et al.* 2003) and two studies showed a weak to modest association with this disorder for (TAA)n and (CTG)n (Prasad *et al.* 2004; Skol *et al.* 2003). In Asian samples, four studies showed no association with the markers in the promoter region and first exon in Japanese subjects (Imai *et al.* 2001; Kaneko *et al.* 2004; Takahashi *et al.* 2003; Ujike *et al.* 2001), and two studies observed no association in Chinese subjects (Fan *et al.* 2002; Takahashi *et al.* 2003). In African-American samples, two studies showed a weak to modest association with these markers (Luo *et al.* 2004; Skol *et al.* 2003). Three follow-up studies have genotyped the marker (TTAT)n in the intron 17 (Fan *et al.* 2002; Kaneko *et al.* 2004; Skol *et al.* 2003), two of

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which reported weak association with schizophrenia (Fan *et al.* 2002; Kaneko *et al.* 2004).

A meta-analysis of the previous association studies that have genotyped the five markers in the initial report (Wei & Hemmings 2000) showed no significant association between schizophrenia and repeat length of alleles of the (TAA)n, (CTG)n or (TTAT)n polymorphisms, or between the disease and specific risk alleles at these polymorphisms or at the SNP1 or SNP2 polymorphisms (Glatt *et al.* 2005). Heterogeneity and stronger evidence of association was observed in family-based studies than in case–control studies. Hence, they suggested that additional large family-based or genomic-controlled studies would be helpful for definitively specifying the role of NOTCH4 haplotypes in risk for schizophrenia. They also pointed out that because the previous studies are concentrated in the polymorphisms in the 5′ promoter region and first exon, additional sites throughout the gene and its flanking regions should be assessed for association with the disorder.

Notch protein was originally discovered as a *Drosophila* neurogenic protein required for correct segregation of epidermal cells from neuronal cell precursors during embryogenesis (Sugaya *et al.* 1997). The Notch pathway is an evolutionarily conserved cell–cell signaling mechanism; one key role of which is to decide the cell's fate, especially during the neural developmental process (Sestan *et al.* 1999). Notch signaling plays a role in postmitotic differentiation of cortical neurons (Sestan *et al.* 1999; Walker *et al.* 2001). Upregulation of Notch activity would either increase the number of interneuronal contacts or result in arrest of neurite growth or retraction of neurites (Sestan *et al.* 1999). Transcripts of the NOTCH4 gene can be detected in the developing nervous system (Uyttendaele *et al.* 1996). As an important neurodevelopment-related gene, NOTCH4 is a potential candidate gene for a neurodevelopment disorder such as schizophrenia (Lewis & Levitt 2002).

Considering the inconsistency of the replication studies and the suggestions of the meta-analysis study, we aimed to study the association between schizophrenia and NOTCH4 using a systematic approach, which scanned the entire genomic region of NOTCH4 gene, in a relatively large family sample of schizophrenia.

Materials and methods

Subjects

The subjects were recruited from two sample collection programs: the Multidimensional Psychopathology Study of Schizophrenia (MPSS) from 1993 to 2001 (Hwu *et al.* 2002) and the Taiwan Schizophrenia Linkage Study (TSLS) (Hwu *et al.* 2005) from 1998 to 2002. A total of 218 families with at least two affected siblings with schizophrenia were used for this study, of which, 86 families were from MPSS and 132 were from TSLS.

The MPSS families were recruited mainly from the Department of Psychiatry, National Taiwan University Hospital and the University-affiliated Taoyuan Psychiatric Center. Data collection was initiated after informed consent had been obtained from the identified study subjects and their families. All the family members were personally interviewed by research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu 1999). The final diagnostic assessment was formulated by integrating the PDA data, and clinical information was obtained from the medical chart records. The final diagnosis used criteria specified by the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) (APA 1994).

The TSLS families were recruited from hospitals all over Taiwan, except the above two institutions. Data collection was initiated after informed consent had been obtained from the identified study subjects and their family members. All the family members were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen *et al.* 1998). The final diagnostic assessment was formulated by integration of the DIGS data and clinical information from the medical chart records by two board-certified research psychiatrists independently. Research diagnosis was made based on DSM-IV criteria. All the data schedules and medical records for subjects with inconsistent diagnoses from these two independent research diagnosticians were evaluated further by the senior researcher (H-G. H.) to achieve final diagnosis. Detailed information about the recruitment procedures has been previously published (Hwu *et al.* 2005). Both projects of sample recruitment have been approved by the ethics committee of National Taiwan University Hospital.

Through the procedures described above and elsewhere, we enrolled 218 multiplex (i.e. at least two affected siblings) schizophrenic nuclear families, incorporating a total of 864 individuals from whom DNA was available. The family structure detailed by number of siblings and parent genotyped is presented in Table 1. Most of the families (83%) had at least four family members genotyped. A total of 461 individuals were diagnosed schizophrenic; the mean age was 34.5 (±9.4) years and 62.5% were men. The mean age at onset was 22.2 (±6.2) years. The mean age of the unaffected subjects was 52.6 (±15.3) years with 47.5% men.

SNP selection and validation

For a systematic approach, we selected evenly dispersed SNPs in the NOTCH4 gene from a public database (http://www.ensembl.org/Homo_sapiens/martview). These SNPs were distributed across the entire genomic region from the 5′ promoter to the 3′ untranslated region. A total of 14 SNPs for NOTCH4 were selected for further validation. A sample subset of 31 trios and one independent individual was used to validate the 14 selected SNPs. Considering the power of further LD testing, we required SNPs to have a minor allele frequency of more than 10% to be genotyped in the full sample.

SNP genotyping

All SNP markers were genotyped using matrix-assisted laser desorption/ionization times of flight mass spectrometry (MALDI-TOF MS). A DNA fragment (100–300 bp) encompassing each SNP site was amplified using the polymerase chain reaction (PCR) GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. After the PCR amplification and neutralization of the deoxynucleotide triphosphate (dNTP) were performed, the primer extension was done by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and the appropriate dideoxynucleotide triphosphate/dNTP mixture. Extension products were differentiated by mass through the MALDI-TOF.

Table 1: Distribution of families by number of siblings and parents genotyped

	Parents genotyped			
Sibs genotyped	0	1	2	Total
1	0	0	2	2
2	9	15	74	98
3	8	78	17	103
4	2	9	2	13
5	0	1	1	2
Total	19	103	96	

Statistical analysis

We used PEDCHECK version 1.1 (O'Connell & Weeks 1998) and UNKNOWN version 5.23 (Terwilliger & Ott 1994) to check for the lineage of the study families and Mendelian inheritance of SNPs, and the allele procedure in SAS/GENETICS release 8.2 (Institute 2002) to test for Hardy–Weinberg equilibrium. Linkage disequilibrium among markers was measured using coefficient D′ (Hedrick 1987), which was used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software (Abecasis & Cookson 2000; Abecasis *et al.* 2000). Both single-point and haplotype association analyses were carried out using TRANSMIT version 2.5.4 (Clayton 1999). Considering a number of families that had only one parent but several sibs, we also performed the analysis using TDT/S-TDT, with a joint sib and parent–child trio statistic (Spielman & Ewens 1998; Spielman *et al.* 1993). All the SNPs were analyzed using a combined Z′ score approach, and the *P* value was calculated using a normal distribution approximation. The number and ratio of transmission to nontransmission (T/NT) was calculated using GENEHUNTER 2.1 (Liang *et al.* 2001). To clarify if the NOTCH4 alleles were subject to a parent-of-origin effect, we used QTDT software to perform an analysis of parent-of-origin effect by comparing whether a maternal or paternal allele is significantly different during transmission (Abecasis *et al.* 2000). To clarify if the NOTCH4 alleles were more relevant to specific subgroups defined by age at onset, we divided the families into two subsets: one was the families with early age at onset if the age at onset of any one of the affected sibling was below 18, and the other was the families without early age at onset if the age at onset of all affected siblings were above 18. Then, TRANSMIT was performed in the two family subsets. Power estimation was calculated using PBAT (Lange *et al.* 2004). We assumed there were 218 families with two probands without missing parents' genotypes, and the type I error was set at 0.01. The additive inherited model was set up with the parameters of disease minor allele frequencies between 0.15 and 0.4 and population prevalence 0.003, based on previous epidemiological study (Hwu *et al.* 1989).

Results

At the stage of SNP validation, seven SNPs for NOTCH4 with average intermarker distance of 5.3 kb met the validation criterion of minor allele frequency of more than 10%. Table 2 gives a detailed description of the validated SNPs.

The single-locus association analysis using TRANSMIT showed that the T allele of rs2071285 and the G allele of rs204993 were significantly preferentially transmitted to affected individuals ($P = 0.035$, 0.0097, respectively; see Table 2). However, the single-locus association analysis using TDT/S-TDT yielded inconsistent results, which showed no significant preferential transmission of all SNPs (see Table 2).

The intermarker LD assessed by coefficient D′ is presented in Table 3. The LD pattern showed two haplotype blocks, one defined by rs2071285-rs204993 ($D' > 0.8$), and the other by rs397081-rs915894-rs915895 ($D' > 0.7$). The haplotype association analysis showed the T-G haplotype of rs2071285-rs204993 had the trend for overtransmission to affected individuals ($P = 0.053$), and the A-A haplotype was significantly undertransmitted ($P = 0.034$). The transmission/nontransmission ratio of A-A haplotype of this haplotype block was 0.89 and that of T-G haplotype was 1.26 (see Table 4). The haplotype association analysis of the rs397081-rs915894-rs915895 block showed negative results (data not shown).

The analyses of a possible parent-of-origin effect using QTDT showed that there were no significant differences in transmission between a maternal or paternal allele of these seven SNPs (data not shown). The single-point and haplotype association analyses of the families with early age at onset showed similar results to those of total families, although not so significant. The analyses of the families without early age at onset showed significant association with the 5′ SNP rs397081 ($P = 0.042$), while the haplotype association analysis showed negative results (data not shown).

The power estimated by PBAT was more than 80% under the conditions of odds ratio of more than 1.65, and minor allele frequency between 0.2 and 0.35. Under odds ratios of 1.2–1.3 and minor allele frequency between 0.15 and 0.4, the power was estimated as 7.2%–26.8%.

Table 2: Detailed description of the validated single nucleotide polymorphisms (SNPs) of NOTCH4 and the single-locus association analysis results

dbSNP ID	Location*	Intermarker distance (kb)†	Polymorphism‡	Minor allele frequency§	Single-locus association		T/NT¶ (ratio)	
					P value (TRANSMIT)	*P* value (TDT/S-TDT)‖	Major allele	Minor allele
rs397081	Promoter	—	T/C	0.16	0.13	0.17/0.38	328/318 (1.03)	60/70 (0.86)
rs915894	Exon 3	2.2	T/G	0.46	0.58	0.32/0.33	333/335 (0.99)	55/53 (1.04)
rs915895	Intron 3	0.2	T/C	0.49	0.90	0.055/0.087	282/288 (0.98)	64/58 (1.10)
rs415929	Exon 4	1.2	T/C	0.17	0.54	0.066/0.081	244/258 (0.95)	160/146 (1.10)
rs3131290	Intron 11	5.9	G/A	0.13	0.26	0.18/0.21	214/213 (1.01)	176/177 (0.99)
rs2071285	Intron 16	2.7	A/T	0.18	0.035#	0.34/0.46	339/343 (0.99)	54/50 (1.08)
rs204993	3′-UTR	24.9	A/G	0.39	0.0097#	0.41/0.32	286/289 (0.99)	80/77 (1.04)

*The single nucleotide polymorphism (SNP) location for NOTCH4 was determined based on the messenger-RNA accession no. NM_004557.

†The intermarker distance was determined based on the genomic contig accession no. NT_007592.

‡Second allele under oblique line (/) is the minor allele.

§Two SNPs, rs915894 and rs915895, were incompatible with Hardy–Weinberg equilibrium ($P = 0.002$, 0.006, respectively).

‖(−/−) indicates the *P* value of major/minor allele.

¶T/NT, the ratio of transmitted to nontransmitted alleles calculated using GENEHUNTER 2.1.

#The overtransmitted alleles for SNPs, rs2071285 and rs204993 were T allele and G allele, respectively.

Table 3: Intermarker D′ value of the seven single nucleotide polymorphisms of NOTCH4 calculated using GOLD

	rs397081	rs915894	rs915895	rs415929	rs3131290	rs2071285	rs204993
rs397081	...						
rs915894	0.71	...					
rs915895	0.76	0.91	...				
rs415929	0.78	0.08	0.06	...			
rs3131290	0.90	0.18	0.32	0.27	...		
rs2071285	0.87	0.87	0.92	0.96	0.91	...	
rs204993	0.80	0.33	0.32	0.10	0.46	0.86	...

Discussion

We found modest evidence for association of schizophrenia with the distal genomic region of NOTCH4 in Taiwanese families, with at least two siblings affected by schizophrenia. The associated region spans about 25 kb, from the distal genomic region of NOTCH4 (rs2071285) to the genomic region of pre-B-cell leukemia transcription factor 2 (PBX2) (rs204993), which also encompasses the genomic region of the G-protein signaling modulator 3 (GPSM3). Hence, association with the genomic region of GPSM3 and PBX2 cannot be ruled out by this study.

For the inconsistent results between two analytic strategies, we provided the following explanation. Different analytic results may result from the dissimilarity of these two programs. TDT handles trios and S-TDT can only be implemented by including sibs where each consisted of both affected and unaffected. If a family with only one parent available and all sibs are affected, this kind of family cannot be analyzed by the TDT/S-TDT (Spielman & Ewens 1998; Spielman *et al.* 1993). On the contrary, TRANSMIT can handle families with one or two parental genotype missing by using a partial score function. Even though some families have only one genotyped parent and several sibs, TRANSMIT can include such families by using all the available offspring's genotypes to reconstruct parental genotypes possibly; therefore, these families will not be discarded totally (Clayton 1999). For example, SNP rs204993, TRANSMIT could include 216 families for analysis, however, TDT used 97 trios and S-TDT used only 117 discordant families. Therefore, we favored the results of TRANSMIT for its robustness.

Table 4: Haplotype association analyses using TRANSMIT

Haplotype rs2071285-rs204993	Haplotype frequency	Chi-square	*P* value	T/NT (ratio)*
A-A	0.59	4.49	0.034†	342/385 (0.89)
A-G	0.23	0.66	0.42	
T-A	0.001	0.88	0.35	
T-G	0.18	3.76	0.053‡	120/95 (1.26)

*T/NT, the ratio of transmitted to nontransmitted alleles calculated using GENEHUNTER 2.1.

†Undertransmitted to affected individuals.

‡Overtransmitted to affected individuals.

Following the suggestions of a prior meta-analysis (Glatt *et al.* 2005), we adopted a systematic approach, choosing SNPs covering the full haplotype block structure of the gene to test the association in a relatively large family sample. These selected markers were not identical to those of previous studies. To facilitate comparison with previous studies of NOTCH4, the relative locations of the markers genotyped in this and previous studies are plotted in Fig. 1.

Our study also showed no significant association with the four SNPs (rs397081, rs915894, rs915895, and rs415929) distributed from the 5′ promoter region to exon 4. This mirrors the conclusion of the prior meta-analysis study, which showed no significant association between schizophrenia and the four markers in the 5′ promoter region and first exon [(TAA)n, (CTG)n, SNP1, and SNP2] (Glatt *et al.* 2005). Our study did not replicate the evidence for association reported by Zhang *et al.* (2004), which studied SNP2 and three functional SNPs from exon 3 to exon 6, and found weak evidence for association of SNP rs520692 (in exon 5) with schizophrenia ($P = 0.017$). However, we found a modest association with the SNPs (rs2071285 and rs204993) distributed from intron 16 to 3′-UTR (untranslated region). Four previous studies have genotyped the marker (TAAT)n in the intron 17 (Fan *et al.* 2002; Kaneko *et al.* 2004; Skol *et al.* 2003; Wei & Hemmings 2000), two of which found a weak association with (TTAT)n [$P = 0.03$ (Fan *et al.* 2002); $P = 0.012$ (Kaneko *et al.* 2004)]. In conclusion, our study showed a probable association between schizophrenia and the distal genomic region of NOTCH4.

There are several reasons for the poor replication of NOTCH4 association. First, the clinical and genetic heterogeneity of schizophrenia should be taken into consideration. Although not associated with the broad phenotype of schizophrenia, two studies reported that the NOTCH4 locus was associated with the age of onset of schizophrenia (Anttila *et al.* 2003; Takahashi *et al.* 2003), and one study reported an association with frontal lobe function in schizophrenia (Wassink *et al.* 2003). Secondly, ethnicity may contribute to the poor replication. A positive association with the proximal region was reported in two African samples (Luo *et al.* 2004; Skol *et al.* 2003), although the sample size in the two studies was not large enough. The lack of association with the proximal region of NOTCH4 is consistent across the results of the Asian samples and this study (Fan *et al.* 2002; Imai *et al.* 2001; Kaneko *et al.* 2004; Takahashi *et al.* 2003; Tochigi *et al.* 2004; Ujike *et al.* 2001). The evidence for association of distal genomic region of NOTCH4 in our study is consistent with the

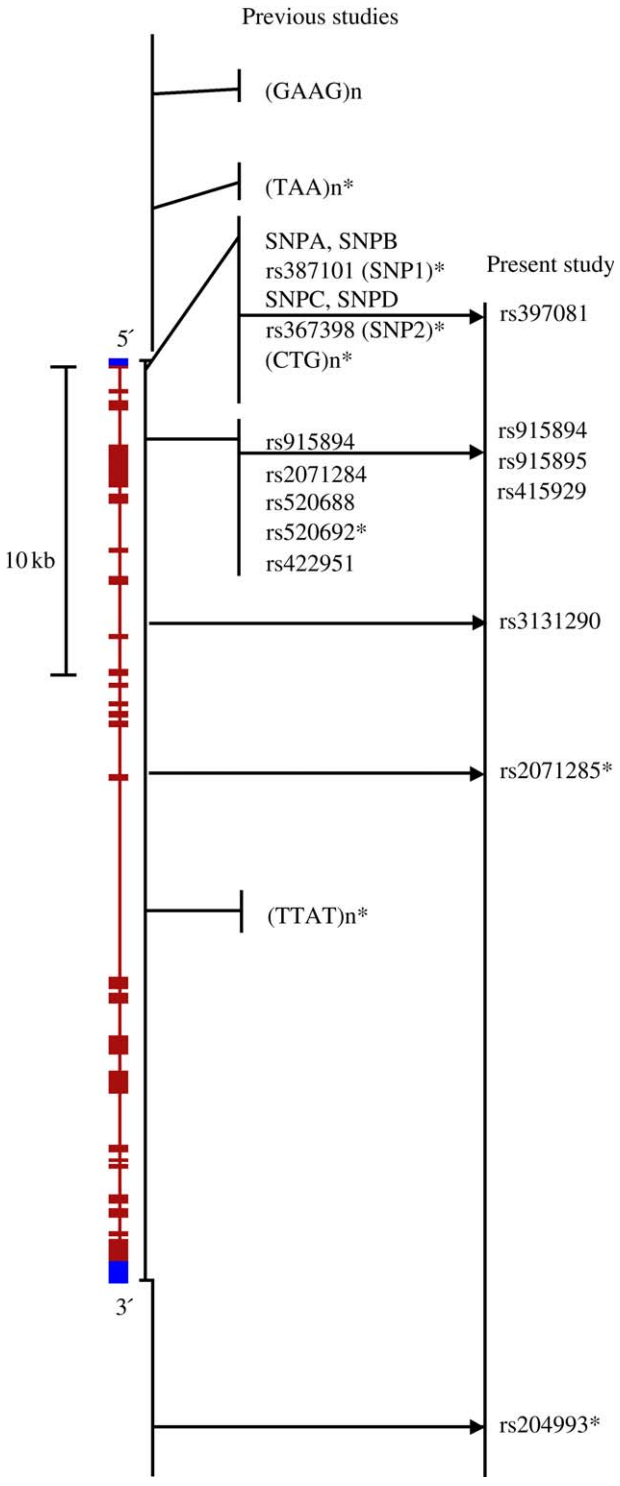

Figure 1: The relative locations of markers of NOTCH4 genotyped in this and previous studies. *Markers that have ever been reported association evidence with schizophrenia in previous studies and in this study. The references for (TAA)n, (CTG)n, (TTAT)n, single nucleotide polymorphism (SNP) 1, and SNP2 are as the review in the paragraphs before Materials and methods section. Additional markers other than the above five markers are listed as follows: (GAAG)n (Prasad *et al.* 2004); (SNP A, B, C, D) (Takahashi *et al.* 2003); rs915894 (Zhang *et al.* 2004), rs2071284 (Tochigi *et al.* 2004), rs520668 (Tochigi *et al.* 2004), rs520692 (Tochigi *et al.* 2004; Zhang *et al.* 2004), rs422951 (Zhang *et al.* 2004).

weak association with (TTAT)n detected by the two Asian studies (Fan *et al.* 2002; Kaneko *et al.* 2004). Finally, as Zhang *et al.* (2004) suggested, there might be two or more disease-underlying variants at the NOTCH4 locus or at a nearby locus, and that the allelic or locus heterogeneity may be one of the possible reasons for the poor replication. Our results might show the phenomenon of either allelic heterogeneity (association with different genomic region of NOTCH4) or locus heterogeneity [association with neighboring tightly linked loci (e.g. PBX2, GPSM3)] in an ethnically distinct sample.

We need to interpret the results with caution for three reasons. First, our analyses suggest association at nominal levels of significance with the distal portion of NOTCH4. None of these results remained significant following corrections for multiple comparisons. Secondly, the results between the two analytic strategies we used were inconsistent. Finally, the power of this study to detect the odds ratios observed using TRANSMIT was inadequate. Therefore, we cannot exclude the possibility of false positive in this study. Further replication for association with the distal genomic region of NOTCH4 and a fine mapping study to delineate the true associated genomic region is warranted.

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