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Family-Based Association Study of *Synapsin II* and Schizophrenia

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***Synapsin II* has been proposed as a candidate gene for vulnerability to schizophrenia on the basis of its function and its location in a region of the genome implicated by linkage studies in families with schizophrenia. We recently reported positive association of *synapsin II* with schizophrenia in a case-control study (Chen et al. 2004). However, since case-control analyses can generate false-positive results in the presence of minor degrees of population stratification, we have performed a replication study in 366 additional Han Chinese probands and their parents by use of analyses of transmission/disequilibrium for three in/del markers and three single-nucleotide polymorphisms. Positive association was observed for *rs2307981* ($P = .02$), *rs2308169* ($P = .005$), *rs308963* ($P = .002$), *rs795009* ($P = .02$), and *rs2307973* ($P = .02$). For transmission of six-marker haplotypes, the global P value was .0000016 (5 degrees of freedom), principally because of overtransmission of the most common haplotype, CAA/−/G/T/C/− (frequency 53.6%; $\chi^2 = 20.8$; $P = .0000051$). This confirms our previous study and provides further support for the role of *synapsin II* variants in susceptibility to schizophrenia.**

Introduction

Functional linkage and association studies suggest that *synapsin II*, located on chromosome 3p25, may be a candidate gene for schizophrenia (MIM 181500). Decreased brain size and increased ventricular volume in patients with schizophrenia (Lawrie and Abukmeil 1998) is best explained by reduced neuropil and neuronal size rather than by a loss of neurons. It may arise from alterations in synaptic, dendritic, and axonal organization—a view supported by immunocytochemical and ultrastructural findings (Harrison 1999). Synapsin II is a neuron-specific phosphoprotein that selectively binds to small synaptic vesicles in the presynaptic nerve terminal (Karlin et al. 2002). It regulates neurotransmitter release from mature nerve terminals and is involved in the formation of new nerve terminals (Greengard et al. 1993). Postmortem studies have shown that the mRNA expression level and protein content of the *synapsin II* gene are significantly reduced in the brains of patients with schizophrenia (Mirnics et al. 2000; Vawter et al. 2002).

Although several linkage studies have shown negative results on chromosome 3 (Schizophrenia Linkage Collaborative Group for Chromosomes 3, 6, and 8 1996; Hovatta et al. 1998; Maziade et al. 2001), a modestly positive LOD score (2.34) was reported on 3p26-24 (Pulver et al. 1995). Significant positive linkage was also found on 3p25.3-p22.1 in a meta-analysis of genome-wide linkage scans of multiplex families with schizophrenia (Lewis et al. 2003). *Synapsin II* is located at the edge of this region.

In our previous work, we examined four markers in *synapsin II* in a sample of 654 cases with schizophrenia and 628 normal controls and reported significant differences in the allele-frequency distribution of SNP *rs795009* ($P = .000018$; odds ratio 1.405; 95% CI 1.202–1.641) between cases and controls (Chen et al. 2004). Moreover, the overall frequency of a four-marker haplotype showed significant differences between cases and controls ($P < .000001$). However, since minor degrees of sample stratification can produce false positives in case-control analyses, we have attempted to validate the role of *synapsin II* by examining transmission in families. To analyze transmission from parents to offspring with schizophrenia in 366 Chinese family trios, we examined—individually and as haplotypes—six markers located in the *synapsin II* gene region.

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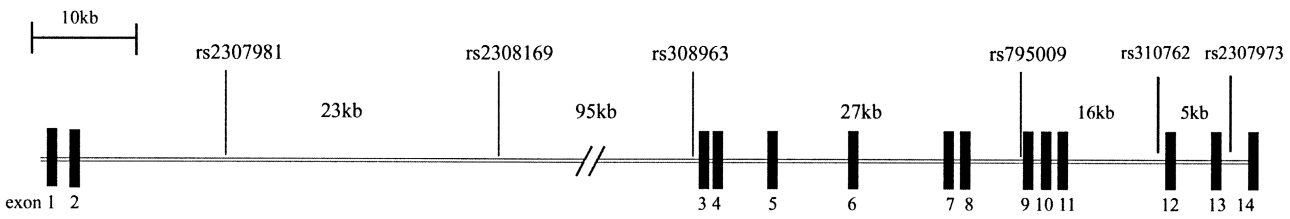

Figure 1 Genomic structure and locations of markers in the human *synapsin II* gene. *Synapsin II* spans >190 kb and is composed of 14 exons. Six markers are indicated by their dbSNP reference ID numbers (see dbSNP Home Page). Distances between the markers are indicated.

Material and Methods

Subjects

DNA samples from 366 Han Chinese family trios, consisting of mothers, fathers, and offspring affected with schizophrenia, were examined. The trios comprised 174 families from Shanghai, 109 from Shanxi province, and 83 from Jilin province. All subjects were of Han Chinese origin. A clinical interview was administered to all probands by a senior psychiatrist. A final diagnosis was made by two independent psychiatrists on the basis of data from the interview and hospital case notes. All probands met DSM-IV criteria for schizophrenia, and individuals with schizoaffective disorder were excluded. All subjects gave informed consent for the genetic analysis, which was reviewed and approved by the ethical committee of the Human Genetics Center in Shanghai. Of the probands with schizophrenia, 181 (49.6%) were men and 195 (50.4%) were women. The mean age at testing was 25.12 years (SD 0.65), and the mean duration of illness was 5 years. Peripheral blood samples were obtained from the subjects for DNA extraction by the phenol-chloroform method.

SNP Genotyping

We studied six markers that were selected from dbSNP (see dbSNP Home Page) (fig. 1). Of these, two in/del markers (*rs2307981* and *rs2308169*) and two SNPs (*rs308963* and *rs795009*) had been studied previously, and one in/del marker (*rs2307973*) and one SNP (*rs310762*) were added for the present study. All were located in the intron/exon region of *synapsin II* and covered ~165 kb. The primers and experiment methods for *rs2307981*, *rs2308169*, and *rs308963* have been described elsewhere (Chen et al. 2004). The marker *rs2307973* was genotyped by MegaBACE 1000 (Amersham Biosciences) with capillary electrophoresis technology. Because of a 2-bp deletion, the products of *rs2307973* were 165 bp and 167 bp. The primers used were 5′-FAM-ACTACGGCAGAGTCACTGTCCC-3′ and 5′-CTGTGAGTCAGAGGCAGAAAGATT-3′.

SNPs *rs795009* and *rs310762* were genotyped with TaqMan technology (Assay-by-Design) on an ABI 7900 system (Applied Biosystems). All probes and primers were designed by the Assay-by-Design service of Applied Biosystems. The standard PCR was performed using the TaqMan Universal PCR Master Mix reagent kit in a 5-μl volume. The primers of *rs795009* are TGTGGAAAGCAACAGTGAACTAAAAT (forward) and CAGTGAGCCACACCAGCAAG (reverse), and the probes are FAM-TGGAAAGTTGCATGCCCT and VIC-TGGAAAGTTTCATGCCCTC. The primers of *rs310762* are TTCCCTCTAAGGTAAGGAGGGTTCT (forward) and CCCAAAGGGAGGGACTTAAAGA (reverse), and the probes are VIC-AGAAACAAACGACGACCC and FAM-AGAAACAAATGACGACCCC.

Statistical Analysis

For single- and multiple-marker haplotype transmission, the program TRANSMIT, version 2.5.4 (Clayton

Table 1

TDT Results Obtained with Six Markers of *Synapsin II*

Marker	Distance[a] (kb)	Genotyping Method	Polymorphism	χ^2	*P*
rs2307981	...	Capillary electrophoresis	−/CAA	5.45	.020
rs2308169	22.7	Capillary electrophoresis	−/ATGCT	7.85	.005
rs308963	117.3	Allele-specific real-time PCR	C/G	9.24	.002
rs795009	144.2	TaqMan	G/T	5.37	.020
rs310762	159.7	TaqMan	C/T	3.85	.050
rs2307973	165.1	Capillary electrophoresis	−/CT	5.18	.023

[a] Distance in kb from *rs2307981*.

Table 2

Allele Frequencies of Markers in *Synapsin II* in Probands and Healthy Controls

Marker	Alleles	No. (Frequency [%]) of Alleles[a] in			*P* (χ^2)	
		New Probands (n = 366)	Previous Cases[b] (n = 654)	Controls[b] (n = 628)	Previous Cases vs. Controls[b]	New Probands vs. Controls
rs2307981	−/CAA	306 (44.2)	630 (48.2)	604 (48.1)	.969 (.001)	.10 (2.68)
rs2308169	−/ATGCT	394 (57.2)	743 (56.8)	715 (56.9)	.95 (.004)	.88 (.02)
rs308963	C/C	222 (32.8)	414 (31.7)	393 (31.3)	.844 (.039)	.47 (.52)
rs795009	T/G	407 (64.2)	719 (55.0)	584 (46.5)	.000018 (18.40)	<.000001 (52.92)
Four-marker haplotype					<.00001 (65.92)	.00000042 (35.21)[c]

[a] Of the first allele shown in each row.
[b] Data from Chen et al. (2004).
[c] Global *P* value analyzed with TRANSMIT (4 df). The overtransmitted haplotype is CAA/−/G/T (P = .0000047; χ^2 = 21.0).

1999), was used. This program uses a score test that is based on the "conditional on parental genotype" likelihood to estimate unknown haplotype phase and missing data. To protect against misleading results caused by rare haplotypes, the command-line instruction "−c3" was used to aggregate all alleles of haplotypes with frequencies <3%. We obtained global *P* values, which estimate the significance of transmission distribution for all of the haplotypes tested, as well as individual *P* values, which estimate the significance of transmission distortion for specific haplotypes. For estimates of the number of degrees of freedom, rare haplotypes were considered together.

We also tested association in a case-control analysis of the 366 probands from the present study versus the controls from our previous study (Chen et al. 2004). The program CLUMP, version 1.6 (Sham and Curtis 1995), was used to compare allele frequencies on the basis of 10,000 simulations. Linkage disequilibrium (LD [D' and r^2]) between two loci was measured using a two-locus LD calculator (Zapata et al. 2001) and EMLD software (see the Programs for Special Needs Web site). An online calculator (see the Calculation of Chi-Square Test for Deviation from Hardy-Weinberg Equilibrium Web site) was used to test for departure from Hardy-Weinberg equilibrium in samples from affected probands and unaffected parents.

Results

All the markers used were in Hardy-Weinberg equilibrium ($\chi^2 < 1.8$). Table 1 lists the results of the transmission/disequilibrium test (TDT) for each marker. Significant differences in transmission were found for five markers: *rs2307981* (P = .02), *rs2308169* (P = .005), *rs308963* (P = .002), *rs795009* (P = .02), and *rs2307973* (P = .02). Table 2 shows significant differences between the allele frequency of marker *rs795009* in the 366 probands of the present study and that of the controls examined in our previous study (Chen et al. 2004). *P* values were <.00001 (χ^2 = 52.92). The four-marker haplotype analysis using TRANSMIT showed that the global *P* value was .00000042 (χ^2 = 35.2 [4 df]), and the overtransmitted haplotype was CAA/−/G/T (P = .0000047). Estimates of LD between pairs of markers are presented in table 3. All estimates of D' were >0.65, and estimates of r^2 were >0.3.

We analyzed the transmission of haplotypes comprising the six markers (the only haplotypes analyzed separately were those with a frequency of at least 3% in either the case or the control group). Rare haplotypes were aggregated in the analysis. There were 26 haplotypes in total, but only 5 showed frequencies >3%. Haplotypes with frequencies >3% accounted for 89% of

Table 3

Estimation of LD between the Six Markers

	LD Estimate (D' or r^2) for Marker Pair					
	rs2307981	*rs2308169*	*rs308963*	*rs795009*	*rs310762*	*rs2307973*
rs2307981	...	.82	.47	.54	.39	.52
rs2308169	.93	...	.47	.57	.41	.55
rs308963	.86	.84	...	.65	.47	.61
rs795009	.85	.86	.87	...	.69	.81
rs310762	.66	.67	.81	.91	...	.63
rs2307973	.85	.85	.84	.91	.87	...

Note.—The standardized D' values are shown below the diagonal, and the r^2 values are shown above the diagonal.

Table 4

Estimation of the Six Marker–Based Haplotype Probabilities and χ^2 Test of Multimarker Haplotypes Using TRANSMIT

Haplotype						Transmissions		Variance (Observed-Expected)[c]	χ^2	*P*
rs2307981	*rs2308169*	*rs308963*	*rs795009*	*rs310762*	*rs2307973*	Observed[a]	Expected[b]			
−	ATGCT	C	G	T	CT	135.4	139.6	48.8	.4	.55
−	ATGCT	G	G	T	CT	20.7	24.7	10.9	1.5	.23
−	ATGCT	G	T	C	−	31.8	36.6	15.5	1.5	.23
CAA	−	G	T	C	−	248.2	212.9	59.6	20.8	.0000051
CAA	−	G	T	T	−	26.9	27.6	9.3	.055	.814

NOTE.—Haplotypes were omitted from the analysis if the estimated haplotype probabilities were <3%. The global χ^2 value is 34.87 (5 df); the global *P* value is .0000016 (5 df).

[a] Observed transmissions of haplotype to affected offspring.

[b] Expected transmissions under Mendelian inheritance.

[c] Variance of observed to expected transmissions.

haplotype diversity. The global *P* value for the TDT of the five common haplotypes plus the rare haplotypes aggregated as a sixth haplotype was .0000016 (χ^2 = 34.87 [5 df]). The most significant individual finding was the overtransmission of the most common haplotype, CAA/−/G/T/C/− (frequency 53.6%; χ^2 = 20.8; *P* = .0000051) (see table 4).

Discussion

In our previous study, we found significant differences between cases and controls in the allele-frequency distribution of SNP *rs795009* (*P* = .000018) (Chen et al. 2004). Although the cases and controls were matched carefully by geography, it is difficult to classify Chinese samples, because the Han Chinese have an extremely long and complex demographic history (starting with the ancient Huaxia tribe in the 21st–8th centuries B.C., which then integrated with numerous tribes and ethnic groups) (Ding et al. 2000; Oota et al. 2002; Shi et al. 2004). One way to overcome population stratification is to use family-based analysis.

In this study, we used the TDT to test six markers, including the four markers used in our previous study (Chen et al. 2004). Five markers showed significant transmission differences. The positive result found in the trios at *rs795009* is supported by the present case-control comparison as well as our previously reported case-control study (Chen et al. 2004). The *P* values of *rs2307981, rs2308169,* and *rs308963* were all >.05 in the case-control study, but these three markers showed positive association with schizophrenia in the present TDT study.

Because all of the D' estimates were >0.65 and all of the r^2 estimates were >0.3, we analyzed the five common six-marker haplotypes and found transmission differences. The most significant difference was the overtransmission of the most common haplotype, CAA/−/G/T/C/− (frequency 53.6%; χ^2 = 20.8; *P* = .0000051), which implies that CAA/−/G/T/C/− may be a risk haplotype for schizophrenia. Because the positive markers are all intronic, they are unlikely to be the direct disease-causing polymorphisms. However, our results taken together lend strong support to the view that at least one susceptibility locus for schizophrenia in Chinese subjects may reside within or nearby the region that spans markers *rs2307981, rs2308169, rs308963,* and *rs795009* in the *synapsin II* gene. In conclusion, our results provide further support for our previous study (Chen et al. 2004) that showed that *synapsin II* may be an important gene in the predisposition to schizophrenia.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Calculation of Chi-Square Test for Deviation from Hardy-Weinberg Equilibrium, http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for schizophrenia)

Programs for Special Needs, http://request.mdacc.tmc.edu/~qhuang/Software/pub.htm (for EMLD software)

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