Candidate gene polymorphisms among North Indians and their association with schizophrenia in a case–control study

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Abstract

Knowledge of candidate gene polymorphisms in a population is useful for a variety of gene–disease association studies, particularly for some complex traits. A number of candidate genes, a majority of them from the monoaminergic pathway in the brain, have been very popular in association studies with schizophrenia, a neuropsychiatric disorder. In this study diallelic/multiallelic polymorphisms in some dopaminergic, serotonergic and membrane-phospholipid-related genes have been evaluated in a control population recruited from North India. Association, if any, of these allelic variants with schizopherenia has been tested using a case–control approach. The case data have been taken from our published family-based association studies in schizophrenia. Of the eight genes tested in this study, association with schizophrenia was observed for only two gene polymorphisms, one in the promoter region of the serotonin 2A receptor gene and the other in the tryptophan hydroxylase gene. One new allele for the dopamine transporter gene (with eight repeats, 570-bp size), not reported in any population so far, has been identified in one individual in our sample. The data generated in this study, besides providing a normative background for various disease association studies, are a significant contribution to the population-specific genome database, a currently growing requirement.

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Introduction

Complex diseases are characterized by an unclear phenotype, high frequency in a population, genetic complexity, and a non-Mendelian mode of inheritance, making identification of susceptibility gene(s)/loci an extremely difficult task. Both linkage and association studies have been performed extensively in these cases. Genetic analysis of complex traits using the linkage approach requires extended families with multiple affected members across generations or a prohibitively large number of affected sibpairs. Further, a large number of samples have to be evaluated to identify genes of modest effect in polygenic (multifactorial) disorders, since the susceptibility conferred by any locus is relatively small (Risch and Merikangas 1996). Association studies, on the other hand, are more popular because of their relative simplicity and economy (Rao *et al.* 1998), especially for identifying susceptibility genes for multifactorial disorders like diabetes, hypertension and schizophrenia. Association studies follow the inheritance of a gene within a population and can be either family-based or case–control. A conventional case–control association study tests if an allele is more often found in individuals with the disease (cases) than in normals (controls). However, a priori

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knowledge of the candidate genes/loci is essential for association studies.

Schizophrenia is a common but complex disorder, with a worldwide lifetime risk of 1% (Gottesman and Shields 1982). It affects more than 2.5 million individuals and has a prevalence of 5.2 per 1000 adults in India (Reddy and Chandrashekar 1998). Clinically, hallucinations and delusions characterize schizophrenia. Cognitive impairment, emotional instability, disordered thought process and social deterioration are its distinct characteristics, and lead to severe disability during the prime of life. On the basis of epidemiological surveys, twin and adoption studies, and family history, a genetic predisposition for the disease has been demonstrated convincingly. Linkage studies using both conventional methods and the more recent genomewide scans suggest putative genes on almost all the chromosomes (see review by Prasad *et al*. 2002). The knowledge of candidate genes for schizophrenia has been contributed largely by biochemical and pharmacological investigations of gene products that have been implicated in disease pathogenesis. Most of the candidate genes are from the dopaminergic and serotonergic pathways in the brain (Carlsson 1988; Tamminga 1998) and also those involved in abnormal metabolism of membrane phospholipids and the phospholipase C signalling pathways.

Earlier, drugs like reserpine were used to treat schizophrenia by reducing the dopamine content of dopamine neurons thus making them less available for synaptic transmissions. Current dopamine theory suggests that excess dopamine yields positive symptoms and vice versa (Matthyse 1973). The neurotransmitter serotonin is involved in regulation of a wide variety of physiological effects such as depression, sleep and body temperature. There is evidence for abnormalities in serotonin neurotransmission in major psychiatric disorders such as schizophrenia and depression. Therefore, allelic variation of genes encoding elements mediating serotonergic neurotransmission may underlie the aetiology of various psychiatric diseases. Neurotransmitters, their receptors, their circuits in the brain, and drug action on the circuits are the key factors investigated for understanding not only schizophrenia but also other complex neurological/ neuropsychiatric disorders such as Parkinson's disease, Alzheimer's disease and bipolar disorder.

A large number of association studies in schizophrenia have been carried out with diallelic and multiallelic polymorphisms of monoaminergic pathway genes (see Prasad *et al*. 2002). For association studies by the commonly used case–control approach, knowledge of candidate gene polymorphisms, their frequencies and Hardy–Weinberg equilibrium in the control population is a prerequisite. Such data would not only provide the normative background for various gene–disease association studies in a sample population but also contribute to developing the genome database for the population. The collection and cataloguing of a large group of polymorphisms will serve as a database of potential disease-associated alleles. The present study was undertaken to investigate polymorphisms and determine the baseline allele frequencies among North Indians for some of the candidate genes implicated in schizophrenia. These data for the control group have been used in case–control analysis of schizophrenia among North Indians. Data on allele frequencies for the cases have been taken from our published family-based studies of schizophrenia (Prasad *et al*. 1999; Chowdari *et al*. 2001; Semwal *et al*. 2001). All the candidate genes investigated in this study, except cytosolic phospholipase A2 (*PLA2G4A*) and inositol polyphosphate-1-phosphatase (*INPP1*), are from the dopaminergic and serotonergic pathways in the brain (figure 1). These include dopamine receptor D3 (*DRD3*), dopamine transporter (*DAT*), catechol-*O*-methyltransferase (*COMT*), serotonin 2A receptor (*HTR2A*), serotonin 2C receptor (*HTR2C*) and tryptophan hydroxylase (*TPH*). Brief descriptions of these genes are given below.

***DRD3, dopamine receptor D3 (chromosomal location 3q13.3)*:** All typical and atypical drugs show a great affinity for dopamine receptor D3 (DRD3). Expression of the *DRD3* gene is restricted to the limbic areas of the brain, the site for emotion and cognition (Sokoloff *et al*. 1990). An A to G *Msc*I (Ser9Gly) polymorphism at position 25 downstream of the start codon in exon 1 (Crocq *et al*. 1992) has been evaluated.

***DAT, dopamine transporter (5p15.3)*:** Reuptake of dopamine into the presynaptic terminal is mediated by dopamine transporter and also leads to inactivation of synaptic DA effects (Jaber *et al*. 1997). Psychostimulant drugs like cocaine block the neurotransmitter transport, thereby aggravating preexisting symptoms in schizophrenic patients, asserting the fact that defective DAT could be involved in aetiology of schizophrenia. A 40-bp VNTR (variable number of tandem repeats) polymorphism in the 3′ untranslated region of the gene has been evaluated.

***COMT, catechol-O-methyltransferase (22q11)*:** The enzyme catalyses the *O*-methylation of catecholamine neurotransmitters (dopamine, adrenaline, noradrenaline) thereby inactivating them. According to the transmethylation hypothesis an abnormality in the methylation of catecholamines might induce some of the symptoms of schizophrenia. Velo-cardio-facial syndrome (VCFS), which presents with features reminiscent of schizophrenia (Bassett and Chow 1999), is caused by microdeletions in a region spanning this gene. A diallelic polymorphism involving G to A transition at codon 158 has been analysed.

***HTR2A, serotonin 2A receptor (13q14)*:** Drugs like clozapine, used in schizophrenia treatment, are known to have

a high affinity for HT2A. Therefore two single-nucleotide polymorphisms, one a T to C change at nucleotide 102 in the coding region of the gene (Warren *et al*. 1993) and the other an A to G change at nucleotide 1438 within the regulatory region of the gene (Spurlock *et al*. 1995) which could influence the expression of the gene and affect receptor density, have been analysed.

***HTR2C, serotonin 2C receptor (Xq24)*:** Alterations in the functional state of serotonin 2C receptors in schizophrenia are suggested by distinct clinical and hormonal responses (Roth and Meltzer 1995). These receptors are synthesized in the brain and have been implicated in enhanced efficacy of atypical antipsychotic drugs, such as clozapine. A G to C transversion at nucleotide 68 of the coding sequence of the gene which gives rise to a cysteine to serine substitution in the N-terminal extracellular domain of the receptor protein (Lappalainen *et al*. 1995) has been investigated.

***TPH, tryptophan hydroxylase (11p15.3)*:** Tryptophan hydroxylase is the rate-limiting enzyme in the biosynthesis of serotonin. It catalyses the biopterin-dependent monooxygenation of tryptophan to 5-hydroxytryptophan (Jequier *et al*. 1969; Kaufman 1987), which is thereafter decarboxylated to the neurotransmitter serotonin. It may be a factor influencing serotonin turnover and behaviour controlled by serotonin for several psychiatric conditions like suicidal tendencies (Nielsen *et al*. 1994, 1998; Mann *et al*. 1997), bipolar disorders (Nielsen *et al*. 1997) and alcoholism (Nielsen *et al*. 1998). An intronic and a promoter polymorphism in the gene have been evaluated in this study.

***PLA2G4A, cytosolic phospholipase A2 (1q25)*:** Increased activity of cytosolic phospholipase A2 results in abnormal metabolism of membrane phospholipids (Horrobin *et al*. 1994; Ross *et al*. 1999) and reduced levels of arachidonic acid and docosahexainoic acid. It preferentially hydrolyses arachidonic acid from the SN-2 position of membrane phospholipids. Elevated PLA2G4A activity in the serum has been reported among patients compared with healthy controls or psychiatric patients without schizophrenia (Gattaz *et al*. 1987, 1990, 1995; Ross *et al*. 1997). A diallelic polymorphism in the first intron of the *PLA2G4A* gene has been analysed.

***INPP1, inositol polyphosphate-1-phosphatase (2q32)*:** Pharmacogenetic factors influence the therapeutic mood-stabilizing effects of lithium (Berridge *et al*. 1982; Atack 1996). Lithium has been shown to directly or indirectly alter activity of several neuronal signalling system components including regulatory G proteins, adenylate cyclase, inositol-phosphate-degrading enzymes, protein kinase C, various ion channels and glycogen synthase kinase-3*b* (see Berridge *et al*. 1989). IPP is a lithium-blockable enzyme of the phospholipase C signalling pathway and therefore its gene is a good candidate. A silent C to A

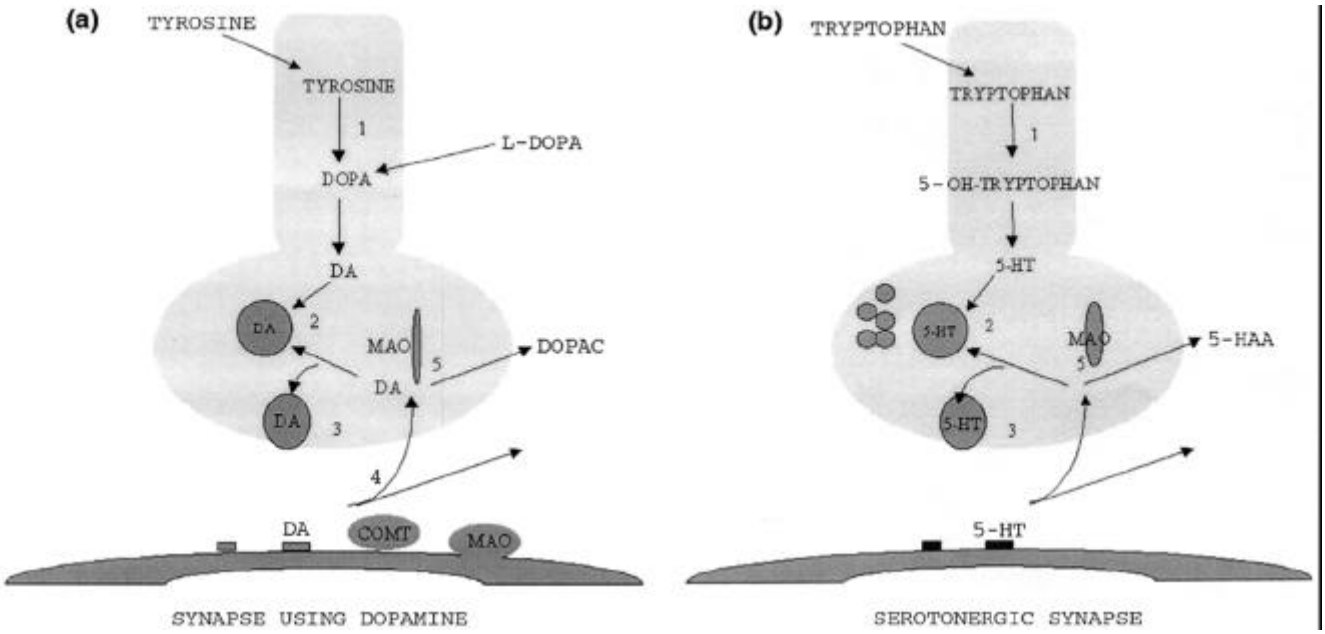

Figure 1. (a) Dopaminergic and (b) serotonergic pathways in the brain. DA in (a) and 5-HT in (b) at the synapse represent dopamine receptor D3 and serotonin receptor respectively. TPH acts at step number 1 in (b). Numbers 1–5 in both (a) and (b) represent the synthesis, storage, release, receptor effect and reuptake of dopamine and serotonin in the respective pathways.

transversion at nucleotide 973 in the coding region of the gene has been evaluated.

Methods

***Controls*:** A total of 936 individuals (497 males and 439 females) were included as controls in this study. Of these, 255 individuals (150 males and 105 females, referred to as group 1 controls) were recruited as controls from the health centres on the University of Delhi campus and from various hospitals in and around Delhi based on a questionnaire and with informed consent. Only individuals without any family history of mental disorders were recruited. The mean age of the participants was 32.9 ± 12.78 years (approximately 10 years older than the average age of schizophrenia cases). The remaining 681 individuals (347 males and 334 females, referred to as group 2 controls) included in the analysis were the unaffected parents of schizophrenia cases recruited for our earlier family-based studies.

***Cases*:** Allele frequency data based on a total of 536 cases (302 males and 234 females) were taken from our published studies (Prasad *et al*. 1999; Chowdari *et al*. 2001; Semwal *et al*. 2001). All the cases (probands) with one or both parents available were recruited from in and around Delhi, and had an average age of 22.87 ± 7.05 years. Diagnosis of the probands was based on an exhaustive structured diagnostic interview (DIGS, diagnostic interview for genetic studies (Nurnberger *et al*. 1994) and FIGS, family interview for genetic studies based on DSM IV (*Diagnostic and statistical manual* of the American Psychiatric Association) criteria as laid down by the National Institute of Mental Health). Informed consent was obtained.

***Genotyping*:** Five mililitres of venous blood was drawn from each of the individuals and genomic DNA was isolated by conventional phenol–chloroform method. Polymerase chain reaction (PCR)-based genotyping was carried out using gene-specific primers, *Taq* polymerase (GIBCO-BRL) and standard PCR protocols. The primer sequences, annealing temperature, restriction enzymes used, method of analysis, and the polymorphisms identified for each of the markers are presented in table 1. The PCR restriction fragment length polymorphism (RFLP) products were run on 2% agarose gel with size marker

Table 1. PCR primers, annealing temperature and method of analysis for the markers analysed.

Marker (reference)	Primer sequence	Ann. temp./time	Restriction enzyme/method
Dopaminergic genes			
DRD3, exon 1 (Crocq *et al*. 1992)	5′-GCTCTATCTCCAACTCTCACA-3′ (forward) 5′-AAGTCTACTCACCTCCAGGTA-3′ (reverse)	55°C/30 sec	*Msc*I/RFLP
DAT, 3′ untranslated region (Vandenberg *et al*. 1992)	5′-TGTGGTGTAGGGAACGGCCTGAGA-3′ (F) 5′-TGTTGGTCTGCAGGCTGCCTGCAT-3′ (R)	71°C/30 sec	40-bp VNTR
COMT, G to A at 158 (Daniels *et al*. 1996)	5′-ACTGTGGCTACTCAGCTGTG-3′ (F) 5′-CCTTTTTCCAGGTCTGACAA-3′ (R)	57°C/30 sec	*Nla*III/RFLP
Serotonergic genes			
HTR2A, gene, T to C at 102 (Warren *et al*. 1993)	5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCC GTCTGCTACAAGTTCTGGCTT-3′ (F) 5′-CTGCAGCTTTTTCTCTAGGC-3′ (R)	60°C/45 sec	*Msp*I/RFLP
HTR2A, promoter, A to G at 1438 (Spurlock *et al*. 1995)	5′-ACTGCGAAACCAACTTATTTCC-3′ (F) 5′-TTGTGCAGATTCCCATTAAGG-3′ (R)	58°C/20 sec	*Hpa*II/RFLP
HTR2C, G to C at 68 (Segman *et al* 1997)	5′-GGCCTATTGGTTTGGCCAT-3′ (F) 5′-CTGCCATGATCACAAGGATG-3′ (R)	57°C/30 sec	*Nla*III/RFLP
TPH, intron 7 (Nielsen *et al*. 1992)	5′-TTCAGATCCCTTCTATACCCCAGAG-3′ (F) 5′-GGACATGACCTAAGAGTTCATGGCA-3′ (R)	64°C/2 min	*Hae*III/SSCP in 220-bp fragment
TPH, promoter, A to G 6526 (Rotondo *et al*. 1999)	5′-TGGCATTGAAGTAAGAGCAC-3′ (F) 5′-GTTTCATGCAGGTATTAGTG-3′ (R)	54°C/30 sec	*Sau*3AI/RFLP
Membrane phospholipid and C signalling pathway genes			
PLA2G4A, intron 1 (Wei *et al*. 1998)	5′-CATGCCCGTAATACCAGCAC-3′ (F) 5′-GCAAACAAGATGAATGGGAAC-3′ (R)	60°C/30 sec	*Ban*I/RFLP
1NPP1, coding region (Steen *et al*. 1998)	5′-CTAGAAGAAACGGCAGTGAAAC-3′ (F) 5′-TAACCAGCAACAGGACAAAG-3′ (R)		*Dde*I/RFLP

and profiles documented with ethidium bromide staining. Polymorphism in the TPH gene intron was evaluated using methods for single-stranded conformation polymorphism (SSCP). The PCR product was resolved on 5% polyacrylamide gel with silver nitrate staining. Briefly, the silver staining method involved fixation of the gel bound to the glass plate in 40% methanol for 10 min; treatment with 8% 2 N HNO_3 for 2 to 3 min followed by a 5-min rinse in water; impregnation of the gel with 0.2% $AgNO_3$ for 30 min and water rinse for 20 s; developing of the gel with developer (3% Na_2CO_3 and 750 µl of 37% formaldehyde) for 2.5 min, followed by a brief rinse in water and 5 min in 2.1% citric acid stop solution. The gel was lifted onto Whatman No. 3 paper, covered with Saran Wrap and dried under vacuum for 90 min.

***Statistical analysis*:** Allele frequencies in the control population were calculated from the genotypes observed for the controls and tested for Hardy–Weinberg equilibrium. Genotypewise distribution data for the cases were taken from our earlier family-based studies (Prasad *et al*. 1999; Chowdari *et al*. 2001; Semwal *et al*. 2001). A c^2 test was used to make case–control comparisons for each of the polymorphisms tested.

Results

Diallelic/multiallelic polymorphism of several candidate genes (listed in table 1) was evaluated in the control North Indian sample. Only the *INPP1* gene did not show any allelic variant in the large sample set and therefore this gene was not included in further analysis. Observed allele frequencies for all the other genes are presented in table 2. These frequencies were calculated for control group 1 and control group 2 separately but no significant difference was observed between the groups (c^2 values ranged from 0.0001 (P = 0.99) to 0.036 (P = 0.9) for different markers with df = 1 for all markers except *DAT* where df = 7. Therefore the frequencies presented in table 2 and used for all further Hardy–Weinberg equilibrium

Table 2. Allele frequencies for markers in controls (this study) and cases (earlier studies) and c^2 values for case–control comparisons.

Marker	No. of controls	Allele frequencies	No. of cases	Allele frequencies	Case–control c^2
*DRD3**	891	1 = 0.57	524	1 = 0.57	0.517, df = 2, P = 0.5
		2 = 0.43		2 = 0.43	
COMT	535	1 = 0.53	262	1 = 0.53	0.19, df = 2, P = 0.7
		2 = 0.47		2 = 0.47	
DAT	580	1 = 0.01	307	1 = 0.01	16.32, df = 35, P = 0.999
		2 = 0.01		2 = 0.01	
		3 = 0.002		3 = 0.002	
		4 = 0.12		4 = 0.12	
		5 = 0.85		5 = 0.85	
		6 = 0.02		6 = 0.02	
		7 = 0.002		7 = 0.002	
		8 = 0.001***		8 = 0.001	
HTR2A (promoter)	700	1 = 0.47	436	1 = 0.41	14.71, df = 2, P = 0.001
		2 = 0.53		2 = 0.59	
HTR2A (gene)	570	1 = 0.44	364	1 = 0.42	1.25, df = 2, P = 0.6
		2 = 0.56		2 = 0.58	
TPH (gene)	440	1 = 0.46	293	1 = 0.34	35.21, df = 2, P = 0.0005
		2 = 0.54		2 = 0.66	
TPH (promoter)	536	1 = 0.41	296	1 = 0.41	2.41, df = 2, P = 0.3
		2 = 0.59		2 = 0.59	
*HTR2C***	242	1 = 0.07	227	1 = 0.09	Females, 0.81, df = 2, P = 0.7
		2 = 0.93		2 = 0.91	Males, 0.12, df = 1, P = 0.8
PLA2G4A	469	1 = 0.38	235	1 = 0.40	0.98, df = 2, P = 0.7
		2 = 0.62		2 = 0.60	

*Genotype frequencies are not in Hardy–Weinberg equilibrium for this marker.
**Allele frequencies for this X-linked gene were the same in both males and females.
***This allele (repeat 8, 570 bp) has only been reported in our sample.

testing and case–control comparisons are based on pooled control samples. Of the total 936 controls, different numbers were analysed for the different markers and these numbers are indicated against each gene in table 2. The genotypes for all the genes except *DRD3* are in Hardy–Weinberg equilibrium (c^2 values ranged from 1.59 ($P = 0.5$) to 4.41 ($P = 0.1$) with df = 2. The total number of cases analysed and the c^2 values obtained for case–control comparisons are also presented in table 2.

In the case of DRD3, a marginal excess of 1–2 heterozygotes and 2–2 homozygotes was observed in the control population ($c^2 = 7.1$; df = 2; $P = 0.05$). In the case of DAT, unlike the diallelic polymorphism in the majority of the markers analysed, multiallelic VNTR polymorphism was observed, with eight different alleles (table 2). Alleles with five repeats (450-bp size) are predominant in the population, followed by alleles with four repeats (410 bp) and then by alleles with six repeats (490 bp). Only one individual with eight repeats (570 bp) was observed. This is the first report of an allele with eight repeats in *DAT* gene from any population. All the genotypes for this marker are in Hardy–Weinberg equilibrium.

Neither allelewise nor genotypewise underrepresentation or overrepresentation was observed among the case sample for all markers except *HTR2A* (promoter polymorphism) and *TPH* (gene polymorphism). In *HTR2A*, a slight decrease of 1–1 and excess of 2–2 homozygotes were observed, which suggest a possible positive association of this marker with disease ($c^2 = 14.71$; df = 2; $P = 0.0005$). An identical pattern was observed for *TPH*, with significantly lower than expected 1–1 homozygotes and excess of 2–2 homozygotes ($c^2 = 35.21$; df = 2; $P = 0.0005$).

Discussion

This study was aimed at establishing the baseline frequencies for the diallelic and multiallelic polymorphisms for several of the monoaminergic genes, and *PLA2G4A* and *INPP1* among a North Indian control sample. Such data are essential for a variety of gene–disease association studies where candidate genes have been implicated. The documented polymorphisms, the first and only report of its kind from India, also contribute to developing the genome database for the North Indian population.

Allele frequencies calculated for control group 1 did not differ significantly from those for control group 2 despite the obvious bias due to recruitment of group 2 individuals through probands. This enabled pooling and consequently provided a large control sample set for analysis. The frequencies calculated on the basis of this substantial sample size are expected to represent the true allele and genotype frequencies in the population.

In family-based association studies, a large number of uninformative families get eliminated during the transmission disequilibrium test (TDT), thus lowering the sample size considerably. However, case–control studies utilize all genotypes obtained for cases and controls but could be plagued by population stratification bias. Comparison of the results from this case–control study with the already published family-based TDT results (available for all markers except *HTR2C*) (Prasad *et al*. 1999; Chowdari *et al*. 2001; Semwal *et al*. 2001) reveals a consensus of no association for all markers except *HTR2A* and TPH. This is probably due to the very small sample size which was finally analysable for these two markers in the family-based TDT analysis. However, despite a large sample size investigated in this study, we would be cautious in our total dismissal of any association of the other tested genes with schizophrenia. With information becoming available on a large number of single nucleotide polymorphisms (SNPs) in candidate genes, an exhaustive SNP analysis in the strong candidate genes analysed in this study seems necessary for identification of disease-associated SNPs or alleles.

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