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Associations between *DRD*s and schizophrenia in a Korean population: multi-stage association analyses

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Abbreviations: DIGS, diagnostic interview for genetic studies; *DRD*s, dopamine receptor genes; DSM-IV, diagnostic and statistical manual of mental disorders-4th edition; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MALDI-TOF MS, matrix-assisted laser desorption-time-of-flight mass spectrometry; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats

Abstract

The dysregulation of the dopaminergic system has been implicated in the pathophysiology of major psychosis, including schizophrenia, with dopamine receptor genes (*DRD*s) presently targeted as the most promising candidate genes. We investigated *DRD1-5* for association with schizophrenia using a multi-stage approach in a Korean sample. One hundred forty-two SNPs in *DRD1-5* were selected from the dbSNP, and the associations of each SNP were then screened and typed by MALDI-TOF mass spectrometry using pooled DNA samples from 150 patients with major psychosis and 150 controls. Each of the suggested SNPs was then genotyped and tested for an association within the individual samples comprising each pool. Finally, the positively associated SNPs were genotyped in an extended sample of 270 patients with schizophrenia and 350 controls. Among the 142 SNPs, 88 (62%) SNPs in our Korean population were polymorphic. At the pooling stage, 10 SNPs (*DRD1*: 2, *DRD2*: 3, and *DRD4*: 5) were identified ($P < 0.05$). SNPs rs1799914 of *DRD1* ($P = 0.046$) and rs752306 of *DRD4* ($P = 0.017$) had significantly different allele frequencies in the individually genotyped samples comprising the pool. In the final stage, with the extended sample, the suggestive association of *DRD4* with rs752306 was lost, but the association of *DRD1* with rs1799914 gained greater significance ($P = 0.017$). In these large-scale multi-stage analyses, we were able to find a possible association between *DRD1* and schizophrenia. These findings suggested the potential contribution of a multi-step strategy for finding genes related to schizophrenia.

Keywords: Asian continental ancestry group; gene pool; genetic association studies; receptors, dopamine; schizophrenia

Introduction

The dopamine hypothesis is the oldest neurochemical theory of the pathophysiology of psychosis (Tost *et al*., 2010). The contemporary pathophysiological model of the dopamine hypothesis assumes that psychotic symptoms are triggered by a dysregulation of dopaminergic activity in the brain (Kapur *et al*., 2005; Tost *et al*., 2010). The dysregulation of the dopamine system has been one of the most enduring and plausible explanations for the pathophysiology of schizophrenia (Abi-Dargham and Moore, 2003; Kapur *et al*., 2005; Seeman *et al*., 2006; Di Forti *et al*., 2007; Talkowski *et al*., 2008; Tost *et al*., 2010). A large body of genetic association studies has report-

ed an association of dopamine receptor genes (*DRD*s) with schizophrenia. However, a single susceptibility gene probably does not exist, even though a few specific variants in *DRD*s have been suggested to play small but significant roles in individual susceptibility to schizophrenia (Dubertret *et al*., 1998; Glatt *et al*., 2003; Jonsson *et al*., 2003; Owen *et al*., 2004; Talkowski *et al*., 2008; Saiz *et al*., 2010; Srivastava *et al*., 2010).

Although the theoretical advantages of an association design in detecting the modest effects expected in a complex disease are apparent (Risch, 2000), most previous association studies of schizophrenia, including those on *DRD*s, have only examined one or a few variants in one or a few genes of interest. Considering the most likely mode of inheritance of schizophrenia is *via* multiple genes, each with a small effect, a limited numbers of polymorphisms and a relatively small sample size for investigation can be significant limitations. Association designs have been suggested as an alternative, with a dense set of single nucleotide polymorphisms (SNPs) covering the regions of interest in multiple genes (Chowdari *et al*., 2007, Hong *et al*., 2010). However, the expense, the time-consuming nature, and the labor involved in such large-scale genotyping may be restrictive factors for individual laboratories (Chowdari *et al*., 2007).

Many researchers have demonstrated the efficacy of DNA pooling and associated technologies that accurately and reliably estimate SNP or microsatellite allele frequencies in a pooled DNA sample as an initial screen for genetic associations (Mohlke *et al*., 2002; Sham *et al*., 2002; Werner *et al*., 2002; Schnack *et al*., 2004). Two- and three-stage association tests have been carried out using many polymorphisms and DNA pooling techniques to find susceptibility loci for schizophrenia (Hoogendoorn *et al*., 2005; McGhee *et al*., 2005; Chowdari *et al*., 2007; Gunnell *et al*., 2007; Zaharieva *et al*., 2008). Hoogendoorn *et al*. performed multi-stage analyses of associations on 18 microsatellite markers in 12 dopaminergic genes including *DRD*s (Hoogendoorn *et al*., 2005). They were able to identify one marker in *DRD5* from pooled allele frequency comparisons, but its significance was lost in analyses with an extended sample. Chowdari *et al*. also performed multi-stage association tests on 69 SNPs in *ACSL6* and *SIRT5* for schizophrenia, which comprised analyses of allele frequencies estimated by DNA pooling, individually genotyped pools, and extended sample genotyping (Chowdari *et al*., 2007). An association study using pooled DNA genotyping could identify susceptibility genes for schizophrenia by screening the 5q31-32 linkage region (Zaharieva *et al*., 2008). Although several studies did not yield convincing evidence for associations between schizophrenia and the genes of interest, the multi-stage strategy proved to be straightforward and particularly helpful for researchers as a rapid and efficient tool in large-scale association studies.

We conducted a comprehensive three-step analysis to investigate the association between five dopamine receptor genes (*DRD1*, *2*, *3*, *4*, and *5*) and schizophrenia in a Korean population. The scheme for analysis was as follows. Stage 1: SNPs in *DRD*s were selected from the dbSNP, and allele frequencies were estimated using pooled DNA analysis using Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). Stage 2: We genotyped the pooled sample individually for screened SNPs and conducted genetic association analyses. Stage 3: Genetic association analyses for suggestive SNPs were performed with an extended sample.

Results

Results of Stage 1 review of SNPs assays tested for association by using DNA pools

Of a total of 142 SNPs, 88 (62%) SNPs in the control sample had minor allele frequencies of 1.5-48.5% and were thus designated as polymorphic. We removed markers with poor quality data and spectra with signal-to-noise ratios below 3.5 or with a peak height below 1.0 intensity unit. We also removed SNPs for which fewer than three of six possible observations remained for any pool. The median standard deviations of estimated allele frequencies in pools of controls and cases were 0.0127 and 0.0138, respectively.

As detailed in Table 1, 10 of 88 SNPs exhibited significantly different frequencies between case (n = 150) and control (n = 150) groups at the 5% level of significance. We identified two SNPs at *DRD1*, three SNPs at *DRD2*, and five SNPs at *DRD4*. These SNPs with a pre-set case-control difference were then genotyped individually among the samples composing the pools using MALDI-TOF MS analysis.

Results of Stage 2 genetic association analyses for suggested SNPs using individual samples from DNA pools

The samples constituting the DNA pools were genotyped individually for 10 SNPs with suggestive associations in Stage 1. When we evaluated the Hardy-Weinberg equilibrium (HWE) in the control

Table 1. Results of association testing of 10 SNPs in *DRD*s using pools of DNA

Gene	Position	Position in bp*	Marker	Allele	Pooled analysis of allele frequencies		*P*-value	Individually genotyped samples of allele frequencies			*P*-value		
					CTR (n = 150)	Cases (n = 150)		CTR (n = 150)	Cases (n = 150)	SZ (n = 117)	CTR vs. Cases	CTR vs. SZ	HWE in CTR
DRD1	Exon1	19680488	rs265981	G/A	78.31	98.12	< 0.0001	91.33	87.67	87.61	0.143	0.160	0.896
	Exon2	19679491	rs1799914	G/A	72.56	84.07	0.0006	84.56	90.00	90.17	0.046	0.056	0.124
DRD2	Promoter	16908767	rs1799978	A/G	70.75	80.57	0.0051	84.00	87.00	88.03	0.297	0.186	0.085
	Intron 1	16900773	rs4245149	G/A	49.18	59.93	0.0082	66.67	60.56	59.17	0.127	0.082	0.035
	Intron 1	16872035	rs4620755	G/A	57.13	69.09	0.0024	52.33	55.33	53.85	0.461	0.728	0.764
DRD4	Promoter	576199	rs936460	T/C	91.26	84.96	0.0170	89.12	90.54	90.00	0.567	0.743	0.528
	Promoter	576433	rs4987059	G/A	98.22	100.00	0.0201	99.00	99.67	99.57	0.316	0.446	0.902
	Promoter	576496	rs936461	G/A	49.07	62.86	0.0007	80.61	82.53	80.26	0.549	0.921	0.004
	Promoter	576784	rs1800955	T/C	78.10	87.98	0.0013	59.00	61.07	60.78	0.605	0.679	0.022
	Intron 1	577622	rs752306	G/A	93.46	83.00	< 0.0001	88.33	81.33	81.62	0.017	0.029	0.448

* Genomic sequence (www.ncbi.nlm.gov, build 127).

Cases include 117 individuals with schizophrenia, 16 with schizophrenia spectrum disorders, and 17 with bipolar I disorder.

SZ: schizophrenia, CTR: control, HWE: Hardy–Weinberg equilibrium

samples, rs4245149 ($P = 0.035$), rs936461 ($P = 0.004$), and rs1800955 ($P = 0.022$) deviated from equilibrium. Because we had used a pooled sample of patients with schizophrenia (n = 117) and other major psychoses (n = 33), we compared the samples in two ways: all cases vs. controls and schizophrenic patients vs. controls. When comparing all cases with controls, allele-wise analyses for a single marker revealed two significant associations at rs1799914 of *DRD1* (chi-square = 3.983, $P = 0.046$, OR = 1.643, CI: 1.006-2.684) and rs752306 of *DRD4* (chi-square = 5.713, $P = 0.017$, OR = 0.575, CI: 0.364-0.909). Analysis of the schizophrenia sample at two SNPs revealed results similar to those found using all cases (rs1799914 of *DRD1*, chi-square = 3.651, $P = 0.056$, OR = 1.675, CI: 0.983-2.853; rs752306 of *DRD4*, chi-square = 4.745, $P = 0.029$, OR = 0.587, CI: 0.362-0.951), though rs1799914 did not show an association at the nominal level of significance (Table 1).

A linkage-disequilibrium (LD) test was carried out on a pair-wise basis for all SNPs in the 117 patients with schizophrenia and the 150 controls. Little evidence for linkage disequilibrium between SNPs in individual genes was observed (Table 2).

Results of Stage 3 genetic association study with suggested SNPs in the extended sample

The SNPs rs1799914 of *DRD1* and rs752306 of

Table 2. Pair-wise LD analyses in *DRD*s using samples of individuals with schizophrenia and controls

		DRD1		*DRD2*			*DRD4*				
		rs265981	rs1799914	rs1799978	rs4245149	rs4620755	rs936460	rs4987059	rs936461	rs1800955	rs752306
DRD1	rs265981		0.0154								
	rs1799914	0.0174									
DRD2	rs1799978				0.1017	0.0101					
	rs4245149			0.0976		0.0472					
	rs4620755			0.0108	0.0487						
DRD4	rs936460							0.0005	0.0000	0.0390	0.0248
	rs4987059						0.0019		0.0011	0.0067	0.0010
	rs936461						0.0000	0.0004		0.0193	0.0169
	rs1800955						0.0102	0.0145	0.0004		0.0019
	rs752306						0.0160	0.0013	0.0198	0.0091	

LD statistics are presented according to r^2 values. Upper and lower diagonals represent the results for patients with schizophrenia (N = 117) and those for controls (N = 150), respectively.

Table 3. Results of association testing of two SNPs suggesting an association with schizophrenia based on previous stages in the extended sample

Marker	HWE *P*-value	Genotype distribution			*P*-value	Allele frequency		*P*-value
rs1799914 of *DRD1*		GG	GA	AA		G	A	
SZ (n = 269)	0.299	0.822	0.164	0.015	0.074*	0.903	0.097	0.017
CTR (n = 350)	0.028	0.751	0.214	0.034		0.859	0.141	
rs752306 of *DRD4*		GG	GA	AA		G	A	
SZ (n = 269)	0.923	0.729	0.249	0.022	0.463	0.853	0.147	0.469
CTR (n = 349)	0.056	0.716	0.244	0.040		0.838	0.162	

* indicates *P*-value of exact test.

SZ: schizophrenia, CTR: control, HWE: Hardy–Weinberg equilibrium.

DRD4 were assayed in an extended sample of 270 patients with schizophrenia and 350 control individuals using the TaqMan genotyping method. The concordance rates of genotypes between MALDI-TOF MS and the TaqMan method were 100% in each SNP when we checked the genotypes of 267 overlapped individuals (117 schizophrenia and 150 controls). As shown in Table 3, the extended sample did not show associations at rs752306 of *DRD4* (allele wise comparison, chi-square = 0.525, *P* = 0.469, OR = 1.122, CI: 0.821-1.534). rs1799914 of *DRD1* revealed a significant association with a lower *P*-value than the previous stage's association result (allele-wise comparison, chi-square = 5.695, *P* = 0.017, OR = 1.540, CI: 1.078-2.199).

Discussion

Pooled DNA analysis is an economical method for large-scale genetic association studies and is particularly useful for rapid screening of large numbers of SNPs (Sham *et al*., 2002; McCarthy *et al*., 2008). Applying this strategy, we performed a multi-stage genetic association study of the SNPs in *DRD1-5*, looking at the suggestive case-control differences from the pooled analysis and then genotyping them in individual samples for confirmation, followed by a replicate analysis with extended samples. The 10 most studied SNPs were not in LD for each gene. This means that those SNPs in some way represent different regions of each gene. Finally, we were able to find evidence for an association (*P* = 0.017) between rs17799914 of *DRD1* and schizophrenia. However, the possibility of a type I error in our result should be noted, as the *P*-value was not adjusted for multiple testing.

The functional significance of the DRD1 receptor for the pathophysiology of schizophrenia has been postulated. Insufficiency of dopamine transmission in the prefrontal cortex contributes to the cognitive deficits observed in patients with schizophrenia, and robust empirical evidence suggests a central role of prefrontal cortex DRD1 receptors in working memory and executive function (Okubo *et al*., 1997; Abi-Dargham *et al*., 2002; Rybakowski *et al*., 2005). Also, altered dopamine neural networks resulting from abnormal interaction of DRD1 and DRD2 in the prefrontal cortex is thought to be linked to the development of psychotic symptoms (Abi-Dargham and Moore, 2003; Tost *et al*., 2010).

The sequence of *DRD1* has been determined and mapped to chromosome 5q35.1 (Grandy *et al*., 1990), and several studies have suggested that the region is linked to schizophrenia (Sklar *et al*., 2004, Zheng *et al*., 2006, Escamilla *et al*., 2007). As mentioned earlier, a study using pooled DNA genotyping reported that several polymorphisms close to the region were associated with schizophrenia (Zaharieva *et al*., 2008). With respect to association studies involving *DRD1* and schizophrenia, variants of rs4532 (-48A/G, recognized by a DdeI restriction cut site) have most often been studied for *DRD1*. Although the SNP has not yet shown a clear association with schizophrenia (Cichon *et al*., 1994; Liu *et al*., 1995; Dmitrzak-Weglarz *et al*., 2006; Zhang *et al*., 2010), a recent meta-analysis reported that the odds ratio of rs4532 in *DRD1* reached 1.18 (95%CI: 1.01-1.38) (Allen *et al*., 2008). Moreover, rs4532 of *DRD1* was reported to have an association with performance on tests (e.g., the Wisconsin Card Sorting Test) in patients with schizophrenia (Rybakowski *et al*., 2005). Very recently, a study using a Spanish population found an association between two individual SNPs (rs11746641 and rs11749676) and haplotypes containing the SNPs in *DRD1* and schizophrenia, especially among men (Hoenicka *et al*., 2010). These findings support the relationship between *DRD1* and the pathophysiology of schizophrenia.

The associations of the most suggestively as-

sociated SNPs on *DRD*s in Stage 1 failed to be replicated in the Stage 2 analyses. The significant association of rs752306 on *DRD4* in Stage 2 was also not replicable in the Stage 3 analyses. These findings suggest the possibility of negative association between other *DRD*s and schizophrenia. Generally, lack of clear association results between *DRD*s and schizophrenia in various studies may be due to genetic heterogeneity, variability in the loci analyzed, and the power and sample size. Therefore, it would be overstated to conclude that *DRD*s were not associated with a risk of schizophrenia, though our analyses show no support for associations with most *DRD*s.

Despite the negative association results at Stage 2 in our analyses, some of the suggestively associated SNPs in Stage 1 have been studied previously in various neuropsychiatric disorders as well as schizophrenia. Since an association between rs1800955 (-521CT) of *DRD4* and schizophrenia was first reported by Okuyama *et al*. (1999), this polymorphism has become one of the most intensively studied SNPs of *DRD4* not only for schizophrenia (Mitsuyasu *et al*., 2001; Jonsson *et al*., 2003; Xing *et al*., 2007), but also for other psychiatric illness such as personality disorders (Okuyama *et al*., 2000; Mitsuyasu *et al*., 2001; Joyce *et al*., 2003) and ADHD (Kirley *et al*., 2004; Bellgrove *et al*., 2005; Yang *et al*., 2008). Overall, results are still inconsistent despite extensive investigations, and recent meta-analyses for associations between the SNP and schizophrenia showed significant effects with odds ratios of 1.15 to 1.22 (Jonsson *et al*., 2003; Allen *et al*., 2008).

Arinami *et al*. isolated and investigated SNP rs1799978 (-241 AG) in the 5-UTR' region of *DRD2* for any relation with schizophrenia (Arinami *et al*., 1997). While they found no association between rs1799978 of *DRD2* and schizophrenia, similar to our present result, this SNP has been given particular attention recently for its relationship to the phenotype of a differential treatment response within a patient group with schizophrenia (Lencz *et al*., 2006; Xing *et al*., 2007), suggesting the possibility of an association between this SNP and sub-phenotypes of schizophrenia.

Several important limitations of this study should be noted. Only known SNPs were selected from a public database without novel SNPs, thus limiting the possible variants of loci in other introns and the variable number of tandem repeat (VNTR) polymorphisms across the *DRD*s. Another limitation is the possibility of false negative associations among the remaining 78 SNPs during the pooled analysis. The difference of allele frequencies in SNPs between the pooling stage and the subsequent individual genotyping might represent intrinsic limitations of the DNA pooling approach, such as reduced power and difficulties ensuring equimolar representation of samples (McCarthy *et al*., 2008).

The case sample set in the pooling stage was composed of patients with non-homogenous diagnostic phenotypes including bipolar I disorder (n = 17, 11%), which might cause confusion in the screening results. However, most cases were patients with schizophrenia (n = 117, 78%) and schizophrenia spectrum disorders (n = 16, 11%). A number of studies have shown that schizophrenia and bipolar I disorder have overlapping genetic backgrounds, clinical phenotypes, and treatment effects by common therapeutic agents (Hill *et al*., 2008; O'Donovan *et al*., 2009). Taken together, these findings suggest that the diagnostic phenotype of our case sample set may be regarded as representative of schizophrenia and of major psychosis. An additional limitation is the deviation of several SNPs from HWE. In the extended control sample, rs1799914 of *DRD1* also deviated according to the nominal level of significance (P = 0.028). However, the degree of disequilibrium was not tremendous, and genotyping error was less likely because of the perfect concordance shown in the re-genotyping of 10% of all samples and between different the genotypic methods in overlapping samples. It is possible that the deviation from HWE in these SNPs occurred by chance as a result of the limited sample size. Therefore, it is unlikely that our significant finding was substantially based on such deviations in the control sample.

In conclusion, we report a possible association between rs17799914 of *DRD1* and schizophrenia. Considering the functional significance of *DRD*s in the hypothesis that the dysregulation of the dopamine system is relevant to the pathophysiology of psychosis, *DRD*s may be implicated in the disease itself or specific behavioral phenotypes seen in schizophrenia and other psychiatric disorders, though our analyses show no support for associations with most *DRD*s. As case-control association studies are vulnerable to methodological artifacts that arise from population stratification, further studies with other ethnic groups are needed to support our results. We performed a multi-stage analytic study to comprehensively examine the genetic association of schizophrenia with the five *DRD*s. Our findings supported the efficacy of a large-scale multi-stage association study method using SNP information from a public database for finding genes linked to schizophrenia.

Methods

Subjects

Written informed consent was obtained from each subject prior to the study. This study was approved by the ethics committee at Seoul National University Hospital.

For the DNA pooling study, blood samples from a total of 150 patients with psychosis and 150 normal controls were randomly selected from our patient sample set with a major psychotic disorder and control sample set, respectively. Of the 150 patients, 117 met the Diagnostic and Statistical Manual of Mental Disorder-4th Edition (DSM-IV) criteria for schizophrenia, and 16 patients had schizophrenia spectrum disorders, including schizoaffective disorder or delusional disorder. The others met criteria for bipolar I disorder (n = 17), and all bipolar I patients had exhibited psychotic features at least once in their lifetimes.

For the extended sample set, a total of 270 patients with schizophrenia (DSM-IV), which included 117 from the pooled set, were recruited for this study. Consensus diagnostic meetings of more than two psychiatrists were held regularly to evaluate the participants' final diagnoses. Most of the patients (196) were comprehensively interviewed by trained nurses using the Korean-translated version of the Diagnostic Interview for Genetic Studies (DIGS) (Nurnberger *et al*., 1994; Joo *et al*., 2004). Exclusion criteria based on additional information garnered from medical records included subjects younger than 18, a history of any kind of organic brain abnormality, an alcohol problem, drug abuse, or other physical conditions presenting with psychiatric symptoms. A total of 350 unrelated Korean normal controls, which included 150 from the pooled set of the subjects, were recruited randomly from healthy volunteers following a brief interview by a psychiatrist. Subjects with past history or current evidence of organic mental disorders, abuse of illegal substances, and any medical conditions that might give rise to mental symptoms were excluded. All subjects with schizophrenia and healthy controls in this study were ethnically Korean.

SNP selection

One hundred forty-two putative SNPs for dopamine receptor genes were selected based on the information of the dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). The function and heterozygosity of SNPs were considered mainly for the inclusion. Coding or regulatory SNPs showing a minor-allele frequency of more than 1% among Asians have been selected. Furthermore, those SNPs within 2 kb upstream of the transcription start site, the first intron and all exonic regions of five genes, *DRD1-5*, were selected. Among the selected 142 SNPs of *DRD*s, eleven SNPs were missense variants (*DRD1*: rs5330, rs5331, *DRD2*: rs1800496, rs1110977, *DRD3*: none, *DRD4*: rs1800443, *DRD5*: rs6282, rs2227843, rs2227852, rs2227847, rs2227842, rs2227851). The first intron sequence for each gene has been investigated since DNA methylation has become an issue in the pathophysiology of psychiatric diseases including schizophrenia and in the mechanism of action of psychotropics (Newton and Duman, 2006; Muir and McKechanie, 2009). The methylation of CpG islands is often involved in tissue-specific gene expression and has been identified in Intron 1 of *DRD4* and *DRD2* (Kamakura *et al*., 1997; Zhang, 2007). These genes span 125.12 kb; information on the exonic structure and transcript details of each gene are available on the AceView website (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). We then amplified and screened these SNPs for heterozygosity in a pooled DNA sample of 300 individuals (pools of 150 cases and 150 controls each) based on SNPs with a minor allele frequency of 1.5-48.5% using MALDI-TOF MS (Sequenom, San Diego, CA). For each case- and control-based pool and each SNP, six allele frequency estimates were calculated, derived from three separate PCR amplifications and two separate panels.

DNA extraction and pool construction

Genomic DNA was extracted from subjects' blood samples. DNA was measured with Fluoroskan Ascent (Thermo Labsystems, Franklin, MA) together with Pico green reagents and kits (P-7589, Molecular Probes, Eugene, OR). The selected DNA samples were diluted to a standard concentration, and individual aliquots of DNA were transferred into a single tube by using the Hamilton ML2200 and Vivace automated pipetting stations to ensure that a constant amount of each DNA sample was transferred to the pool. The pool was then mixed gently and requantitated before further dilution to a working concentration of 5 ng/μl.

PCR, MassEXTEND (SEQUENOM, San Diego) reactions

For all assays of allelotyping or genotyping processed in this stage, the same conditions were used. The MALDI-TOF MS assay was performed under standard conditions set forth by Buetow *et al*. (2001) with little modification. PCR amplification was performed in a total volume of 5 μl containing Genomic DNA (25 ng for pooled and 2.5 ng for individual), 0.5 units of Taq polymerase (HotStarTaq, Qiagen, Valencia, CA), 25 μmol of each dNTP (Promega, Madison, WI), 50 pmol of gene-specific PCR primer 1 and 2 carrying a universal sequence tag, and 10 pmol of universal sequence primer using 384-well microtiter plates. Cycling was performed in a Thermocycler (model 9600; Perkin-Elmer; Branchburg, NJ) at 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 30 s, and a final extension of 3 min at 72°C.

A shrimp alkaline phosphatase (SAP) reaction was performed to dephosphorylate unincorporated dNTPs. The temperature conditions were 37°C for 10 min followed by the inactivation of SAP at 85°C for 5 min.

Subsequently, homogenous MassEXTEND reactions (hME™, SEQUENOM) were performed by using triple terminator mixes. Assays were grouped according to SNP-specific requirements on the termination mixes (ddACG, ddACT, ddAGT, and ddCGT, respectively). The final reaction volume of 9 μl comprised 1 unit of Thermosequenase (Amersham Pharmacia, Piscataway, NJ), 50 μM of the respective termination mix (ddTTP, ddATP, ddCTP, ddGTP), and 20 pmol of the test-specific MassEXTEND primer. A universal temperature program was applied, comprising an initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 5 s, 52°C for 5 s, 72°C for

5 s, and finally 72°C for 1 min. Primer extension products were resolubilized from the solid support by applying an ammonium hydroxide solution to the beads. For the subsequent MALDI-TOF MS analysis, a 15-nl sample was transferred from a 384-well microtiter plate serially onto patches of a 384-element silicon chip preloaded with 3-hydroxy-picolinic-acid (3-HPA) (SpectroCHIP, SEQUENOM).

MALDI-TOF MS analysis and allele frequency determination

Application of the analyte solution to the preloaded matrix patches of the silicon chip dissolved the matrix. After the solution evaporated, homogenous crystals of matrix and analyte formed. An automated analysis of these samples was performed on a SEQUENOM-Bruker MassARRAY mass spectrometer by scanning the 384 elements with a 337-nm laser pulse. Twenty shots were summed per element to yield the final spectra.

Mass spectra were processed by proprietary software (SPECTROTYPER, SEQUENOM) using baseline correction, peak identification, and peak area calculation algorithms. Normalized peak areas were computed as individual peak areas divided by the sum of the total peak area.

Genotype assays for individual samples of extension

After suggestive SNPs for association with schizophrenia were identified from analyses using the individual genotyping method (Stage 2), we performed individual genotyping with the extended sample (270 schizophrenia sample and 350 controls) for suggestive SNPs by TaqMan™ assays (Applied Biosystems, Foster City, CA). A total reaction volume of 5 μl included 50 ng of template DNA, 2.5 μl 2 × TaqMans™ Universal PCR Master Mix, and 0.5 μl 20 × SNP Genotyping Assay Mix (including primers and fluorescently labeled probes). PCR cycling was performed using an ABI Prisms 7900HT SDS (Applied Biosystems) under the following conditions: 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The PCR was followed by allelic discrimination using the ABI Prisms 7900HT SDS. A confirmatory process was adopted; specifically, 10% of all samples were re-genotyped by the same method.

Statistical analysis in individually genotyped samples

Allele and genotype associations for single markers were evaluated with contingency chi-square tests and exact tests. The pair-wise LD statistic r^2 was calculated with unphased genotype data, and the general pattern of LD was investigated in both the schizophrenia group and the control group. All the genetic data analyses including the HWE evaluation were performed by PowerMarker Version 3.25 (Liu and Muse, 2005). The odds ratio and confidence interval in comparison with allele frequencies were obtained using SPSS Version 12.0 (SPSS, Inc., Chicago, IL).

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References

Abi-Dargham A, Mawlawi O, Lombardo I, Gil R, Martinez D, Huang Y, Hwang DR, Keilp J, Kochan L, Van HR, Gorman JM, Laruelle M. Prefrontal dopamine D1 receptors and working memory in schizophrenia. J Neurosci 2002;22: 3708-19

Abi-Dargham A, Moore H. Prefrontal DA transmission at D1 receptors and the pathology of schizophrenia. Neuroscientist 2003;9:404-16

Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ, Tanzi RE, Bertram L. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nat Genet 2008; 40:827-34

Arinami T, Gao M, Hamaguchi H, Toru M. A functional polymorphism in the promoter region of the dopamine D2 receptor gene is associated with schizophrenia. Hum Mol Genet 1997;6:577-82

Bellgrove MA, Hawi Z, Lowe N, Kirley A, Robertson IH, Gill M. DRD4 gene variants and sustained attention in attention deficit hyperactivity disorder (ADHD): effects of associated alleles at the VNTR and -521 SNP. Am J Med Genet B Neuropsychiatr Genet 2005;136B:81-6

Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, Little DP, Strausberg R, Koester H, Cantor CR, Braun A. High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proc Natl Acad Sci USA 2001;98:581-4

Chowdari KV, Northup A, Pless L, Wood J, Joo YH, Mirnics K, Lewis DA, Levitt PR, Bacanu SA, Nimgaonkar VL. DNA pooling: a comprehensive, multi-stage association analysis of ACSL6 and SIRT5 polymorphisms in schizophrenia. Genes Brain Behav 2007;6:229-39

Cichon S, Nothen MM, Rietschel M, Korner J, Propping P. Single-strand conformation analysis (SSCA) of the dopamine D1 receptor gene (DRD1) reveals no significant mutation in patients with schizophrenia and manic depression. Biol Psychiatry 1994;36:850-3

Di Forti M, Lappin JM, Murray RM. Risk factors for schizophrenia--all roads lead to dopamine. Eur Neuropsychopharmacol 2007;17(Suppl 2):S101-7

Dmitrzak-Weglarz M, Rybakowski JK, Slopien A, Czerski PM, Leszczynska-Rodziewicz A, Kapelski P, Kaczmarkiewicz-Fass M, Hauser J. Dopamine receptor D1 gene

-48A/G polymorphism is associated with bipolar illness but not with schizophrenia in a Polish population. Neuropsychobiology 2006;53:46-50

Dubertret C, Gorwood P, Ades J, Feingold J, Schwartz JC, Sokoloff P. Meta-analysis of DRD3 gene and schizophrenia: ethnic heterogeneity and significant association in Caucasians. Am J Med Genet 1998;81:318-22

Escamilla MA, Ontiveros A, Nicolini H, Raventos H, Mendoza R, Medina R, Munoz R, Levinson D, Peralta JM, Dassori A, Almasy L. A genome-wide scan for schizophrenia and psychosis susceptibility loci in families of Mexican and Central American ancestry. Am J Med Genet B Neuropsychiatr Genet 2007;144B:193-9

Glatt SJ, Faraone SV, Tsuang MT. Meta-analysis identifies an association between the dopamine D2 receptor gene and schizophrenia. Mol Psychiatry 2003;8:911-5

Grandy DK, Zhou QY, Allen L, Litt R, Magenis RE, Civelli O, Litt M. A human D1 dopamine receptor gene is located on chromosome 5 at q35.1 and identifies an EcoRI RFLP. Am J Hum Genet 1990;47:828-34

Gunnell D, Lewis S, Wilkinson J, Georgieva L, Davey GS, Day IN, Holly JM, O'Donovan MC, Owen MJ, Kirov G, Zammit S. IGF1, growth pathway polymorphisms and schizophrenia: a pooling study. Am J Med Genet B Neuropsychiatr Genet 2007;144B:117-20

Hill SK, Harris MS, Herbener ES, Pavuluri M, Sweeney JA. Neurocognitive allied phenotypes for schizophrenia and bipolar disorder. Schizophr Bull 2008;34:743-59

Hoenicka J, Garrido E, Ponce G, Rodríguez-Jiménez R, Martínez I, Rubio G, Jiménez-Arriero MA, Palomo T. Sexually dimorphic interaction between the DRD1 and COMT genes in schizophrenia. Am J Med Genet B Neuropsychiatr Genet 2010;153B:948-54

Hong JM, Kim TH, Kim HJ, Park EK, Yang EK, Kim SY. Genetic association of angiogenesis-and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head. Exp Mol Med 2010;42:376-85

Hoogendoorn ML, Bakker SC, Schnack HG, Selten JP, Otten HG, Verduijn W, van der Heijden FM, Pearson PL, Kahn RS, Sinke RJ. No association between 12 dopaminergic genes and schizophrenia in a large Dutch sample. Am J Med Genet B Neuropsychiatr Genet 2005;134B:6-9

Jonsson EG, Sedvall GC, Nothen MM, Cichon S. Dopamine D4 receptor gene (DRD4) variants and schizophrenia: meta-analyses. Schizophr Res 2003;61:111-9

Joo EJ, Joo YH, Hong JP, Hwang S, Maeng SJ, Han JH, Yang BH, Lee YS, Kim YS. Korean version of the diagnostic interview for genetic studies: validity and reliability. Compr Psychiatry 2004;45:225-9

Joyce PR, Rogers GR, Miller AL, Mulder RT, Luty SE, Kennedy MA. Polymorphisms of DRD4 and DRD3 and risk of avoidant and obsessive personality traits and disorders. Psychiatry Res 2003;119:1-10

Kamakura S, Iwaki A, Matsumoto M, Fukumaki Y. Cloning and characterization of the 5'-flanking region of the human dopamine D4 receptor gene. Biochem Biophys Res Commun 1997;235:321-6

Kapur S, Mizrahi R, Li M. From dopamine to salience to psychosis--linking biology, pharmacology and phenomenology of psychosis. Schizophr Res 2005;79:59-68

Kirley A, Lowe N, Mullins C, McCarron M, Daly G, Waldman I, Fitzgerald M, Gill M, Hawi Z. Phenotype studies of the DRD4 gene polymorphisms in ADHD: association with oppositional defiant disorder and positive family history. Am J Med Genet B Neuropsychiatr Genet 2004;131B:38-42

Lencz T, Robinson DG, Xu K, Ekholm J, Sevy S, Gunduz-Bruce H, Woerner MG, Kane JM, Goldman D, Malhotra AK. DRD2 promoter region variation as a predictor of sustained response to antipsychotic medication in first-episode schizophrenia patients. Am J Psychiatry 2006;163:529-31

Liu K, Muse SV. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 2005;21:2128-9

Liu Q, Sobell JL, Heston LL, Sommer SS. Screening the dopamine D1 receptor gene in 131 schizophrenics and eight alcoholics: identification of polymorphisms but lack of functionally significant sequence changes. Am J Med Genet 1995;60:165-71

McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, Hirschhorn JN. Genome-wide association studies for complex traits: consensus, uncertainty, and challenges. Nat Rev Genet 2008;9:356-69

McGhee KA, Morris DW, Schwaiger S, Nangle JM, Donohoe G, Clarke S, Meagher D, Quinn J, Scully P, Waddington JL, Gill M, Corvin A. Investigation of the apolipoprotein-L (APOL) gene family and schizophrenia using a novel DNA pooling strategy for public database SNPs. Schizophr Res 2005; 76:231-8

Mitsuyasu H, Hirata N, Sakai Y, Shibata H, Takeda Y, Ninomiya H, Kawasaki H, Tashiro N, Fukumaki Y. Association analysis of polymorphisms in the upstream region of the human dopamine D4 receptor gene (DRD4) with schizophrenia and personality traits. J Hum Genet 2001; 46:26-31

Mohlke KL, Erdos MR, Scott LJ, Fingerlin TE, Jackson AU, Silander K, Hollstein P, Boehnke M, Collins FS. High-throughput screening for evidence of association by using mass spectrometry genotyping on DNA pools. Proc Natl Acad Sci USA 2002;99:16928-33

Muir W, McKechanie A. Can epigenetics help in the discovery of therapeutics for psychiatric disorders, especially schizophrenia? Expert Opin Drug Discov 2009;4: 621-627

Newton SS, Duman RS. Chromatin remodeling: a novel mechanism of psychotropic drug action. Mol Pharmacol 2006;70:440-3

Nurnberger JI, Blehar MC, Kaufmann CA, York-Cooler C, Simpson SG, Harkavy-Friedman J, Severe JB, Malaspina D, Reich T. Diagnostic interview for genetic studies. Rationale, unique features, and training. NIMH Genetics Initiative. Arch Gen Psychiatry 1994;51:849-59

O'Donovan MC, Craddock NJ, Owen MJ. Genetics of psychosis: insights from views across the genome. Hum

Genet 2009;126:3-12

Okubo Y, Suhara T, Sudo Y, Toru M. Possible role of dopamine D1 receptors in schizophrenia. Molecular Psychiatry 1997;2:291-2

Okuyama Y, Ishiguro H, Toru M, Arinami T. A genetic polymorphism in the promoter region of DRD4 associated with expression and schizophrenia. Biochem Biophys Res Commun 1999;258:292-5

Okuyama Y, Ishiguro H, Nankai M, Shibuya H, Watanabe A, Arinami T. Identification of a polymorphism in the promoter region of DRD4 associated with the human novelty seeking personality trait. Mol Psychiatry 2000;5:64-9

Owen MJ, Williams NM, O'Donovan MC. The molecular genetics of schizophrenia: new findings promise new insights. Mol Psychiatry 2004;9:14-27

Risch NJ. Searching for genetic determinants in the new millennium. Nature 2000;405:847-56

Rybakowski JK, Borkowska A, Czerski PM, Kapelski P, Dmitrzak-Weglarz M, Hauser J. An association study of dopamine receptors polymorphisms and the Wisconsin Card Sorting Test in schizophrenia. J Neural Transm 2005;112:1575-82

Saiz PA, Garcia-Portilla MP, Arango C, Morales B, Arias B, Corcoran P, Fernandez JM, Alvarez V, Coto E, Bascaran MT, Bousono M, Fananas L, Bobes J. Genetic polymorphisms in the dopamine-2 receptor (DRD2), dopamine-3 receptor (DRD3), and dopamine transporter (SLC6A3) genes in schizophrenia: data from an association study. Prog Neuropsychopharmacol Biol Psychiatry 2010;34:26-31

Schnack HG, Bakker SC, van 't Slot R, Groot BM, Sinke RJ, Kahn RS, Pearson PL. Accurate determination of microsatellite allele frequencies in pooled DNA samples. Eur J Hum Genet 2004;12:925-34

Seeman P, Schwarz J, Chen JF, Szechtman H, Perreault M, McKnight GS, Roder JC, Quirion R, Boksa P, Srivastava LK, Yanai K, Weinshenker D, Sumiyoshi T. Psychosis pathways converge *via* D2high dopamine receptors. Synapse 2006; 60:319-46

Sham P, Bader JS, Craig I, O'Donovan M, Owen M. DNA Pooling: a tool for large-scale association studies. Nat Rev Genet 2002;3:862-71

Sklar P, Pato MT, Kirby A, Petryshen TL, Medeiros H, Carvalho C, Macedo A, Dourado A, Coelho I, Valente J, Soares MJ, Ferreira CP, Lei M, Verner A, Hudson TJ, Morley CP, Kennedy JL, Azevedo MH, Lander E, Daly MJ, Pato CN. Genome-wide scan in Portuguese island families identifies 5q31-5q35 as a susceptibility locus for schizophrenia and psychosis. Mol Psychiatry 2004;9:213-8

Srivastava V, Deshpande SN, Thelma BK. Dopaminergic pathway gene polymorphisms and genetic susceptibility to schizophrenia among north Indians. Neuropsychobiology 2010;61:64-70

Talkowski ME, Kirov G, Bamne M, Georgieva L, Torres G, Mansour H, Chowdari KV, Milanova V, Wood J, McClain L, Prasad K, Shirts B, Zhang J, O'Donovan MC, Owen MJ, Devlin B, Nimgaonkar VL. A network of dopaminergic gene variations implicated as risk factors for schizophrenia. Hum Mol Genet 2008;17:747-58

Tost H, Alam T, Meyer-Lindenberg A. Dopamine and psychosis: theory, pathomechanisms and intermediate phenotypes. Neurosci Biobehav Rev 2010;34:689-700

Werner M, Sych M, Herbon N, Illig T, Konig IR, Wjst M. Large-scale determination of SNP allele frequencies in DNA pools using MALDI-TOF mass spectrometry. Hum Mutat 2002;20:57-64

Xing Q, Qian X, Li H, Wong S, Wu S, Feng G, Duan S, Xu M, Gao R, Qin W, Gao J, Meng J, He L. The relationship between the therapeutic response to risperidone and the dopamine D2 receptor polymorphism in Chinese schizophrenia patients. Int J Neuropsychopharmacol 2007;10:631-7

Yang JW, Jang WS, Hong SD, Ji YI, Kim DH, Park J, Kim SW, Joung YS. A case-control association study of the polymorphism at the promoter region of the DRD4 gene in Korean boys with attention-deficit hyperactivity disorder: evidence of association with the -521 C/T SNP. Prog Neuropsychopharmacol Biol Psychiatry 2008;32:243-8

Zaharieva I, Georgieva L, Nikolov I, Kirov G, Owen MJ, O'Donovan MC, Toncheva D. Association study in the 5q31-32 linkage region for schizophrenia using pooled DNA genotyping. BMC Psychiatry 2008;8:11

Zhang AP, Yu J, Liu JX, Zhang HY, Du YY, Zhu JD, He G, Li XW, Gu NF, Feng GY, He L. The DNA methylation profile within the 5'-regulatory region of DRD2 in discordant sib pairs with schizophrenia. Schizophr Res 2007;90:97-103

Zhang C, Fang Y, Xie B, Cheng W, Du Y, Wang D, Yu S. No genetic association between dopamine D1 receptor gene and [early onset] schizophrenia. Psychiatry Res 2010; 177:350-3

Zheng Y, Wang X, Gu N, Feng G, Zou F, Qin W, Zhang J, Lin W, Tao R, Qian X, He L. A two-Stage linkage analysis of Chinese schizophrenia pedigrees in 10 target chromosomes. Biochem Biophys Res Commun 2006;342:1049-57