Am. J. Hum. Genet. 71:1296–1302, 2002

A Highly Significant Association between a COMT Haplotype and Schizophrenia

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Several lines of evidence have placed the catechol-O-methyltransferase (COMT) gene in the limelight as a candidate gene for schizophrenia. One of these is its biochemical function in metabolism of catecholamine neurotransmitters; another is the microdeletion, on chromosome 22q11, that includes the COMT gene and causes velocardiofacial syndrome, a syndrome associated with a high rate of psychosis, particularly schizophrenia. The interest in the COMT gene as a candidate risk factor for schizophrenia has led to numerous linkage and association analyses. These, however, have failed to produce any conclusive result. Here we report an efficient approach to gene discovery. The approach consists of (*i*) a large sample size—to our knowledge, the present study is the largest case-control study performed to date in schizophrenia; (*ii*) the use of Ashkenazi Jews, a well defined homogeneous population; and (*iii*) a stepwise procedure in which several single nucleotide polymorphisms (SNPs) are scanned in DNA pools, followed by individual genotyping and haplotype analysis of the relevant SNPs. We found a highly significant association between schizophrenia and a COMT haplotype ($P = 9.5 \times 10^{-8}$). The approach presented can be widely implemented for the genetic dissection of other common diseases.

Introduction

Schizophrenia (MIM 181500) is a common psychiatric disease that affects ~1% of the world population. Since genetic and molecular factors have not been identified, schizophrenia is diagnosed by phenotypic symptoms only. Schizophrenia, like most psychiatric disorders, is a complex disease that cannot be explained by a single genetic or environmental factor (McGuffin et al. 1995). Although many monogenic disease genes have been cloned, this has not been the case for genes that increase risk for complex diseases, including schizophrenia. Linkage studies have provided suggestive evidence for many schizophrenia-susceptibility loci, including some on chromosome 22, but there has been little success in the replication of these findings (Pulver 2000). Association studies, mainly with candidate genes, have also not provided any consistent result.

Velocardiofacial syndrome (VCFS [MIM 192430]) is associated with a microdeletion on chromosome 22q11. Patients with VCFS have a high risk of developing psychiatric diseases, especially schizophrenia. Some studies of patients with VCFS have found that 20%–30% of them developed schizophrenia (Murphy et al. 1999). In addition, patients with schizophrenia have an increased rate of the 22q11 microdeletion, compared with the general population (Karayiorgou et al. 1995; Usiskin et al. 1999). These findings suggest that the 1.5–3-Mb 22q11 deletion region contains one or more genes that contribute to schizophrenia risk.

The catechol-O-methyltransferase (COMT [MIM 116790]) gene, which is located in the 22q11 microdeletion, is a candidate gene for schizophrenia that has been studied extensively. COMT is one of the enzymes that degrade catecholamines, including dopamine (Axelrod and Tomchick 1958). The biological functions of COMT, in addition to the gene's location in the microdeletion, make it an attractive candidate gene for schizophrenia.

Received July 31, 2002; accepted for publication August 28, 2002; electronically published October 25, 2002.

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0002-9297/2002/7106-0006$15.00

A nonsynonymous SNP at codon 108/158 in the COMT gene generates a valine-to-methionine (Val/Met) substitution and apparently influences the enzyme's activity (Lachman et al. 1996). However, association studies on the Val/Met polymorphism have failed to produce any conclusive results. Recently, the Val allele was shown to be associated with cognitive characteristics that are present in schizophrenia—namely, poor performance in tests of working memory and inefficiency of information processing in the prefrontal cortex (Egan et al. 2001).

In light of the inconclusive genetic evidence, we tested the possible association of COMT with schizophrenia, by applying a more powerful approach. We tested several SNPs and haplotypes in the COMT gene on a very large sample of an Israeli Ashkenazi Jewish population. The use of the Ashkenazi Jewish founder population has several advantages (Shifman and Darvasi 2001; Zak et al. 2001), primarily in reducing genetic variance and avoiding false-positive results due to population stratification.

Subjects and Methods

Study Subjects

A research group of psychiatrists from seven medical centers in Israel collected samples and data from their hospitalized patients. All diagnoses were assigned by a standard procedure. This procedure included a direct interview using the structured clinical interview for personality disorders (SCID), a questionnaire with inclusion and exclusion criteria and cross-references to medical records. The inclusion criteria specified that subjects had to be diagnosed with one of the schizophrenia subtypes defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), that all four grandparents of each subject were of Ashkenazi Jewish ethnic origin, and that each subject or the subject's legal representative has signed the informed-consent form. The exclusion criteria excluded subjects diagnosed with at least one of the following disorders: psychotic disorder due to a general medical condition, substance-induced psychotic disorder, mood disorder with psychotic features, schizoaffective disorder, schizophreniform disorder, schizotypal disorder, schizoid disorder, and paranoid personality disorder. Corresponding institutional review boards and the National Genetic Committee of the Ministry of Health approved the studies. Samples from healthy Ashkenazi individuals were collected from volunteers in blood banks. Samples from Ashkenazi patients with other diseases, which served as additional controls in the present study, were also collected in Israel under standards similar to those used for the patients with schizophrenia.

Genotyping Assay

Genomic DNA was prepared from blood samples through use of the Nucleon kit (Pharmacia). DNA was diluted to 100 ng/μl, according to optical-density measurements. Pools were created with equal aliquots of each sample. For the amplification of the DNA fragment containing each SNP, one biotinylated and one unmodified primer were designed, using Primer3 (Primer3 Web site), to give an average product of 150 bp. HotStar *Taq* polymerase (Qiagen) was used in 45 amplification cycles of PCR (20 s at 94°C, 30 s at 55°C, and 30 s at 72°C). Genotyping was performed by the Pyrosequencing assay, using an internal primer designed by the Pyrosequencing program and the commercial kit, according to the manufacturer's standard protocol. Results of the pooled DNA samples were analyzed by the Pyrosequencing allele-frequency quantification software.

Statistical Analysis

To measure linkage disequilibrium (LD) between SNPs, we estimated the haplotype frequencies through use of the expectation-maximization algorithm on 70 individual genotypes and calculated Lewontin's D' and the Pearson correlation (Devlin and Risch 1995; Slatkin and Excoffier 1996). Association between markers and schizophrenia risk was tested by comparing genotype and allele frequencies in patients and control individuals, using a standard χ^2 test under a normal approximation. We tested for differences across all possible haplotype frequencies through use of a likelihood-ratio statistic that was calculated from the estimated haplotype-frequency likelihoods (Zhao et al. 2000; Fallin et al. 2001). We calculated the overall significance across male and female 2×2 tables by means of the Cochran-Mantel-Haenszel test, and we estimated the common odds ratio (OR) by means of the Mantel-Haenszel estimator.

Population attributable risk (PAR) was calculated for the tested diplotypes as $(K - 1)/K$, where $K = \sum f_i \times g_i$, f_i is the frequency of the i genotype, and g_i is the estimated genotype relative risk of the i genotype (Khoury et al. 1993). The number of affected sib pairs required to detect linkage to the COMT gene was estimated according to the model of Risch and Merikangas (1996) and using the rs737865-rs165599 haplotype and a multiplicative model with genotypic relative risks of γ and γ^2 for the AG-AG and GG-GG genotypes, respectively.

Results

Estimation of Allele Frequencies in DNA Pools and LD between SNPs

To test the COMT-schizophrenia association, we examined 12 SNPs in the COMT gene (fig. 1). These SNPs,

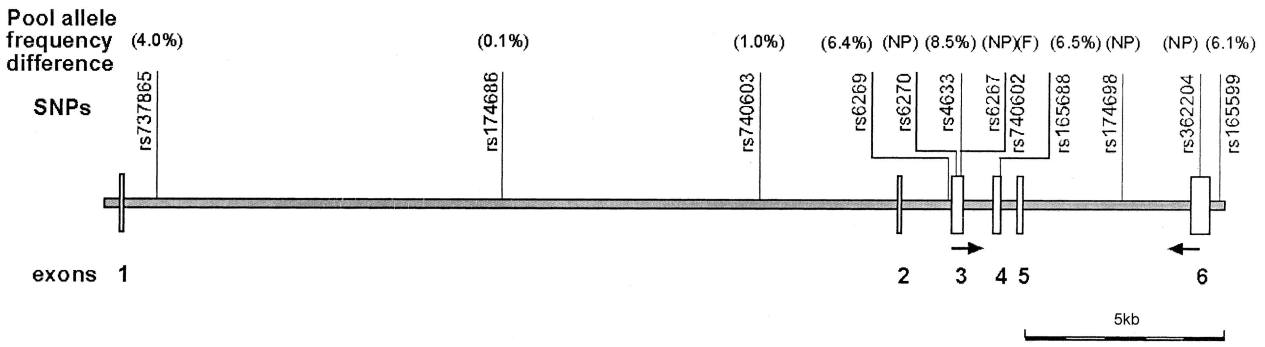

Figure 1 Location of SNPs studied in the COMT locus and allele-frequency differences observed between patients with schizophrenia and control individuals by means of analysis of the DNA pools. Four SNPs were not polymorphic (NP), and one amplification failed (F). Three of the SNPs studied (rs6270, rs6267, and rs165688) cause a nonsynonymous change.

chosen from the public databases (dbSNP Home Page), included all nonsynonymous SNPs, including the well-studied Val/Met polymorphism, rs165688. The 12 SNPs were initially examined by employing DNA pooling. In this process, equal amounts of DNA from hundreds of individuals are mixed together, and the allele frequency is estimated from the relative signals of the alleles in the genotyping assay. For every SNP, we compared the allele frequencies in a total of seven replicated pools comprised of ~300 schizophrenia and 1,000 control DNA samples, using the quantitative Pyrosequencing technology. Individual genotyping of several known pools indicates that allele frequencies in the DNA pools are accurately estimated (Wasson et al. 2002).

Of the 12 SNPs studied, 4 were found not to be polymorphic in the population we studied, and an additional SNP failed to undergo PCR amplification. Among the remaining seven SNPs, we found significant allele-frequency differences between patients and control individuals for five SNPs (see fig. 1). Upon individual genotyping of 70 individuals chosen randomly for each of these SNPs, we found SNPs rs6269 and rs4633 to be in complete LD ($D' = 1$) with SNP rs165688. Therefore, SNPs rs165688 (encoding the Val/Met polymorphism), rs165599 (near the 3′ UTR), and rs737865 (near exon #1) were selected for large-scale individual genotyping.

Genetic Association with Schizophrenia by Individual Genotyping

We initially genotyped the well-studied Val/Met polymorphism (SNP rs165688) in 720 patients with schizophrenia and 2,970 control individuals (table 1). A modest association was found between SNP rs165688 and schizophrenia in men, with the highest significance for the Val/Val (G/G) genotype ($P = .0074$; OR = 1.35; 95% CI 1.08–1.67). Although women did not show an effect, the difference between the effect in men and that in women was not significant ($P = .07$).

SNP rs165599 was next genotyped in 724 patients with schizophrenia and 4,014 control samples (table 1). Surprisingly, we found significant differences in allele and genotype frequencies between men and women among the healthy control individuals (A allele frequency = 65.4% in women vs. 61.3% in men; $P = .00089$). We retested this interesting result by genotyping an independent control sample set of 532 male and 353 female individuals with five different diseases (insulin-dependent diabetes mellitus, non–insulin-dependent diabetes mellitus, prostate cancer, asthma, and colon cancer; ~180 samples for each disease). Essentially the same allele-frequency difference between men and women was observed in this additional control group (A allele frequency = 64.9% in women vs. 59.4% in men; $P = .020$). Overall, the allele difference between men and women is highly significant ($P = 9.0 \times 10^{-5}$).

In light of the results presented above, case and control individuals were matched for gender in all analyses. The effect of SNP rs165599 was found to be highly significant in women, both at the allele level (G allele predisposing, $P = 9.1 \times 10^{-6}$) and at the genotype level, with the G/G genotype displaying the greatest significance ($P = 6.8 \times 10^{-6}$; OR = 2.13; 95% CI 1.52–2.97). Men showed a smaller effect in the same direction ($P = .10$ for the G allele and $P = .09$ for the G/G genotype). The difference between the G/G genotype effect in men and women was significant ($P = .01$).

Lastly, SNP rs737865 was analyzed in 714 patients with schizophrenia and 2,849 control individuals. SNP rs737865 was found to be significantly associated with schizophrenia in men ($P = .0011$) and women ($P = .012$). Male patients with schizophrenia displayed an excess of the G/G genotype ($P = 2.3 \times 10^{-4}$; OR = 1.58; 95% CI 1.24–2.02), whereas female patients showed an

Table 1

Individual Genotyping Results and Association Significance

SNP and Sex	Sample Size		Frequency (%) G		G/G		A/G		A/A		*P*[a]		
	Control	Patient	Control	Patient	Control	Patient	Control	Patient	Control	Patient	Genotype	Allele	G/G
rs165688:													
F	706	262	50.8	53.4	25.9	26.3	49.7	54.2	24.4	19.5	.25	.30	.90
M	2,264	458	52.1	55.8	25.8	31.9	52.6	47.8	21.6	20.3	.027	.041	.0074
All											.041	.024	.023
rs165599:													
F	1,380	263	34.7	44.9	11.7	22.1	45.9	45.6	42.3	32.3	.000010	.0000091	.0000068
M	3,519	461	39.0	41.8	14.8	17.8	48.4	47.9	36.8	34.3	.20	.10	.090
All											.000030	.000088	.000072
rs737865:													
F	685	250	38.7	45.0	15.5	17.6	46.4	54.8	38.1	27.6	.012	.014	.43[b]
M	2,164	464	40.9	45.6	15.8	22.8	50.2	45.5	34.0	31.7	.0011	.0083	.00023
All											.00016	.00036	.00048

[a] *P* values are provided for testing genotype, allele, and G/G versus A/G + A/A frequencies in the patients against those in the control individuals.

[b] In women, a stronger *P* value was obtained when testing A/G and G/G versus A/A ($P = .0029$).

excess of A/G and G/G ($P = .0029$; OR = 1.61; 95% CI 1.18–2.22). Although the allele frequencies in male and female patients with schizophrenia were similar, the genotype distribution was significantly different between the sexes ($P = .052$).

Haplotype Analyses

We tested the haplotype-frequency differences between the patients with schizophrenia and control individuals. We analyzed the three combinations of two-SNP haplotypes, as well as the three-SNP haplotype (table 2). When we compared the overall frequency differences across all possible haplotypes, the rs737865-rs165599 haplotype gave the most significant overall association with schizophrenia ($P = 1.42 \times 10^{-4}$). Since the G allele in the three SNPs was found to be associated with schizophrenia, we specifically tested the association of G-G or G-G-G haplotypes in the four combinations (table 2). The G-G-G haplotype gave the most significant association with schizophrenia ($P = 9.5 \times 10^{-8}$; OR = 1.46; 95% CI 1.36–1.57). Although the G-G haplotype of SNPs rs737865 and rs165599 gave a lower significance ($P = 9.6 \times 10^{-7}$; OR = 1.41; 95% CI 1.32–1.51), the effect of this haplotype is not significantly different from that of the G-G-G haplotype.

PAR and the Power of Association

Although "risk" genes for common diseases are expected to be of weak effect, they may correspond to a high PAR, because of their high allele frequency. The female PAR calculated for the joint genotype of SNPs rs165599 and rs737865 is 32.2%, and the male risk is 13.5%. Thus, 32.2% of female patients with schizophrenia and 13.5% of male patients would not have been affected if the population were monomorphic for the protective alleles of the two SNPs. If the causative alleles are not in complete LD with the haplotype analyzed, then the PARs calculated here are lower limits for the true effect of the gene.

Association studies have been shown to be more powerful than linkage studies in the identification of disease alleles with small genetic effect (Risch and Merikangas 1996). We calculated the number of affected sib pairs that would have been required in order to identify the COMT gene as a schizophrenia-risk gene by means of linkage analysis. The genotypic relative risk for the rs737865-rs165599 haplotype is $\gamma = 1.65$ for women and $\gamma = 1.31$ for men. Thus, the number of affected sib pairs that would have been required is 16,000–190,000, depending on the sex ratio among the samples.

Discussion

The Involvement of the Val/Met Polymorphism in Schizophrenia Susceptibility

Using 12 SNPs distributed in the 27 kb of the COMT gene and testing 7 of them with a very large sample of Ashkenazi Jews, we have been able to confirm a complex association between the COMT gene and schizophrenia. In addition, the data also suggest that SNP rs165688 itself, encoding the Val/Met polymorphism, has only a modest or no effect on schizophrenia risk, which explains the repeated failure by previous investigators to consistently associate COMT with schizophrenia. The

Table 2

Estimated Haplotype Frequencies and Association Significance

	Frequency (%)				*P*			LD[a]	
	Male		Female						
Haplotype	Patient	Control	Patient	Control	Male	Female	All	*r*	*D′*
rs737865-rs165599:					.0093	.0014	.00014	.30	.32
G-A	18.53	17.84	17.08	18.96					
G-G	28.30	23.12	28.74	19.67	.0015	.000055	.00000096		
A-A	38.90	43.50	38.43	45.30					
A-G	14.27	15.55	15.76	16.06					
rs737865-rs165688:					.017	.046	.0057	.68	.85
G-A	3.20	3.12	3.04	2.19					
G-G	43.61	37.71	42.98	36.85	.0016	.021	.000092		
A-A	40.30	44.59	42.76	46.32					
A-G	12.88	14.58	11.22	14.64					
rs165599-rs165688:					.096	.0012	.0011	.44	.58
G-A	6.73	7.69	10.49	7.74					
G-G	34.97	30.69	34.35	27.28	.011	.0024	.00013		
A-A	37.48	40.22	36.07	41.47					
A-G	20.81	21.40	19.08	23.51					
rs165688-rs737865-rs165599:					.0084	.0069	.00045		
G-G-A	15.77	15.83	14.80	17.87					
G-G-G	27.84	21.90	28.18	19.01	.00023	.000047	.000000095		
G-A-A	5.56	5.74	4.49	5.64					
G-A-G	7.33	8.82	6.74	9.14					
A-G-A	2.94	1.96	2.05	1.60					
A-G-G	.26	1.15	.99	.54					
A-A-A	33.33	38.07	34.20	38.95					
A-A-G	6.97	6.54	8.56	7.23					

[a] r = Pearson correlation; D' = standardized LD coefficient.

modest effect occasionally observed at the Val/Met polymorphism is most probably due to the correlation between this SNP and SNP rs737865, which does have a significant effect on susceptibility to schizophrenia. This is implied by the higher OR of SNP rs737865 (OR = 1.58 for rs737865 vs. 1.35 for rs165688) and by the high degree of LD between the two SNPs ($D' = 0.85$; see table 2). Our results also suggest the possibility of more than one functional polymorphism affecting susceptibility to schizophrenia at the COMT locus. This is supported by the finding that SNP rs165599 affects primarily women, whereas rs737865 affects both sexes, although in different ways.

Sex-Specific Susceptibility to Schizophrenia

We report two conceptually different results for SNP rs165599—that is, (*i*) the allele difference between men and women in the general population and (*ii*) the highly significant association of this polymorphism with schizophrenia. Each of these two observations is striking on its own. Although it is not within the scope of the current work, a connection between the two findings should be studied further. For example, one may hypothesize that the two findings are related, in that the G allele, which increases the risk of schizophrenia in women, may also reduce viability of a female fetus. Transmission ratio distortion (TRD) among offspring of different sexes has been reported in mice and humans (Naumova and Sapienza 1994; Naumova et al. 1998; Shendure et al. 1998; Herrmann et al. 1999; Silva et al. 1999). In mice, lethality of male embryos and meiotic drive influenced by the sex chromosomes has been reported as a mechanism of offspring sex-specific TRD (de la Casa-Esperon et al. 2000; Pardo-Manuel de Villena et al. 2000). For humans, this represents, to our knowledge, the first highly significant, replicated result of its kind.

Our findings are suggestive of a sex-specific genetic component in schizophrenia. Sex-specific genetic effects have not been reported elsewhere for this disorder. For example, a large twin study failed to find evidence of sex-specific genetic effects for schizophrenia (Cannon et al. 1998), perhaps reflecting the low statistical power of that approach. Sex differences in the schizophrenic phenotype itself have been described elsewhere (Salem and Kring 1998). A sex-specific genetic component has been demonstrated in obsessive-compulsive disorder (OCD), in which the low-activity COMT allele (Met/Met genotype) is associated with the disease in male patients only (Karayiorgou et al. 1997). The sex-specific phenotypic differences observed in patients with schizo-

phrenia may reflect partial sex-based distinctions in the etiology of the disease, and these may be related to differences in COMT activity. In this regard, it has been shown that women generally have 20%–30% lower COMT activity levels than men (Boudikova et al. 1990), and COMT-knockout mice display sex differences in phenotype (Gogos et al. 1998).

We may speculate that a candidate for the connection between COMT and sex-specific phenotypic differences is estrogen (Salem and Kring 1998), which downregulates COMT transcription (Xie et al. 1999). Accordingly, both symptom exacerbation (Salem and Kring 1998) and COMT activity in schizophrenic women have been observed to parallel blood estrogen levels (Parvez et al. 1978). Finally, female patients with OCD display the converse picture as compared with patients with schizophrenia, with respect to the effects of estrogen (Casas et al. 1986; Neziroglu et al. 1992).

Optimal Study Design

In the present study, we have established a stepwise process that can be widely applied to test candidate genes and even to conduct full genome scans for the identification of the genetic basis of complex traits. The process consists of (*i*) examining a significant number of SNPs per gene and estimating allele frequencies in case versus control individuals, through use of DNA pools, which will ensure that either the functional SNPs, or at least some SNPs that are in LD with the functional polymorphisms are selected; (*ii*) establishing LD patterns, to select an informative subset of SNPs for further individual genotyping; (*iii*) performing individual genotyping in a large sample set with the selected SNPs (large samples are necessary, since one may expect more than one functional polymorphism—or, as shown here, a sex effect that may effectively reduce sample size); and (*iv*) analyzing haplotypes, to extract the maximum statistical power that the data can provide.

In addition to the stepwise procedure described above, the use of a well-characterized homogeneous population, like Ashkenazi Jews (Wijsman 1984; Motulsky 1995; Escamilla 2001; Ostrer 2001), also contributed to the unprecedented level of statistical significance achieved in the present study. The use of homogeneous populations increases gene effect and reduces the chance of false positives due to population stratification. False positives will arise when case and control individuals are not perfectly matched and when the SNPs studied display population allele-frequency differences, as is the case for the Val/Met polymorphism, which displays population allele-frequency differences within and between geographic areas (Palmatier et al. 1999).

Our results strongly associate the COMT gene and schizophrenia, but the possibility that an additional gene in this region confers susceptibility to the disease cannot be excluded. Nonetheless, the combination of previous functional evidence presented for COMT in the context of schizophrenia (Weinberger et al. 2001) and the discoveries elaborated in the present study certainly point toward COMT as a susceptibility gene for schizophrenia.

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

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 [MIM 181500], VCFS [MIM 192430], and COMT [MIM 116790])
Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

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