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Association Study of Schizophrenia and the Dopamine D3 Receptor Gene Locus in Two Independent Samples

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Using a case-control design, an association of schizophrenia with the dopamine D3 receptor gene (D3RG) locus was investigated. Initial analysis of pooled results from published studies revealed a significant excess of individuals homozygous for either allele among the patients. The association was next tested in two cohorts ascertained independently at Pittsburgh, Pennsylvania and at Houston, Texas. The Pittsburgh sample was comprised of patients with schizophrenia (DSM-III-R) (n = 130). The controls belonged to two groups: adults screened for the absence of substance abuse or major psychiatric illness (n = 128), and neonates (n = 160). Multivariate analysis suggested an association with allele 1 of the biallelic D3RG polymorphism in comparison with the adult, but not the neonatal, controls. The association was most marked among Caucasian patients with a family history of schizophrenia (odds ratio 13.69, confidence intervals 1.80, 104.30). Survival analysis suggested an earlier age of onset among male patients homozygous for allele 2. The Houston cohort included Caucasian patients with schizophrenia or schizoaffective disorder (DSM-III-R criteria, n = 50), and normal controls matched for gender (n = 51). In this group, no significant associations were noted among all the patients or among subgroups of patients based on family history or age of onset. Possible reasons for the discordant results are discussed.

KEY WORDS: schizophrenia, dopamine receptor gene, case-control study, association

INTRODUCTION

Family, adoption, and twin studies suggest a significant genetic component in the etiology of schizophrenia [Gottesman and Shields, 1982]. Indeed, the heritability of schizophrenia has been estimated at 71% [Rao et al., 1981]. Nevertheless, the mode of inheritance, as well as the nature of the genetic factors, are unknown. Segregation analysis suggests that monogenic forms of inheritance may not explain the observed familial aggregation adequately [McGue et al., 1983; Carter and Chung, 1980]; on the other hand, a polygenic/multifactorial threshold model does so [Gottesman and Shields, 1967; Ritsner et al., 1992]. Therefore, case-control association studies may be used to identify disease susceptibility genes [Cooper and Clayton, 1988; Bodmer, 1987].

Two groups of investigators, based in Cardiff, U.K. and Rouffach, France, have independently reported an association of schizophrenia with the D3RG locus [Crocq et al., 1992]. The biallelic restriction fragment length polymorphism (RFLP) used in these studies is present in the first exon of D3RG and is identifiable by enzymatic digestion with the endonuclease *BalI* [Lannfelt et al., 1993]. In each study, schizophrenia was associated with increased *homozygosity* for both D3RG alleles. The proposed association is of considerable interest, because D3RG is a favored "candidate gene" in the etiology of schizophrenia [Sokoloff et al., 1990].

Using an enlarged cohort, the Cardiff group subsequently detected significant associations among patients who demonstrated a positive clinical response to antipsychotic drugs, and also among patients with a

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family history of psychiatric illness [Owen et al., 1993; Mant et al., 1993]. An Italian group reported an association between allele 1 and delusional disorder [DiBella et al., 1993]. A Swedish group detected nonsignificant trends for an excess of homozygosity among patients who responded well to antipsychotic drugs [Jonsson et al., 1993]. Our initial studies involving individuals of Caucasian ethnicity did not detect an association with the homozygous state. However, further analysis suggested an association with allele 1 among patients having first or second degree relatives with schizophrenia: there were significant differences in the distribution of genotypes, as well as an excess of allele 1 among these patients in comparison with adult controls screened for psychotic illness [Nimgaonkar et al., 1993]. In contrast, six other groups did not detect an association with the D3RG locus (Table I). Two studies (not listed in Table I) did not detect a significant association at this locus, but used other restriction enzymes to identify D3RG polymorphisms [Cagle et al., 1993; Sabate et al., 1994].

Overall, there appears to be substantial evidence for and against the association. In order to synthesize these results, it is necessary to explain both the discrepancies in the positive results as well as the negative findings. Among the positive studies, the homozygote excess for *both* alleles among patients noted by Crocq et al. [1992] is difficult to understand. Analogous examples of homozygote excess have been noted in association studies of rheumatoid arthritis and uremia [Beckman and Frohlander, 1990]. The homozygote excess has been attributed to typing error, assortative mating, or heterozygote advantage [Morell, 1993; Crocq et al., 1992]. Another plausible reason is that an association with allele 1 of D3RG exists in schizophrenia, but that the expected excess of allele 1 could not be detected in these cohorts, due to a relatively small sample. In support of this, an excess of allele 1 was noted among the patients in the initial Cardiff cohort [Crocq et al., 1992]. A similar excess of allele 1 was noted by two other groups [DiBella et al., 1993; Nimgaonkar et al., 1993]. Furthermore, the association of allele 1 with delusional disorder [DiBella et al., 1993] (Table I) suggests that the findings in schizophrenia may be shared with other psychotic disorders. On the other hand, an association of bipolar disorder with the D3RG locus was not detected [Rietschel et al., 1993].

There are several possible reasons for the negative results. First, though all the studies used identical diagnostic criteria for patients, there could still be subtle phenotypic differences between the different groups of patients (e.g., different syndromic clusters, variations in familiality, age of onset, or drug responsiveness). Since most of the studies listed in Table I did not provide details of the population of patients from which the sample was drawn, this possibility cannot be ruled out.

Second, the sample sizes in these studies may be inadequate. For example, our cohort had a power of 0.8 to detect the association reported in the initial Cardiff study [Crocq et al., 1992]. Thus, a "type II" statistical error cannot be ruled out entirely in the negative studies, which involved cohorts of similar size. In this context, the relatively large variances in the estimates of allele frequencies are noteworthy (Table I). Larger samples are required to demonstrate an association with an allele which is common in the control population (as is the case with allele 1 of D3RG), than with a rare allele [Cox and Bell, 1989].

A third possible explanation for the negative findings is the wide variation among the controls in the different studies. The selection criteria for the controls included married-in members of families seeking genetic counseling [Owen et al., 1993], hospital staff [Crocq et al., 1992; Nothen et al., 1993; Jonsson et al., 1993], or community samples [Nimgaonkar et al., 1993]. This variation may explain the range of mean allele frequencies noted among the controls (Table I). Furthermore, not all the studies screened the controls for the absence of schizophrenia. Indeed, only one study used controls who had passed the age of risk for schizophrenia [Cagle et al., 1993].

TABLE I. Review of D3RG Association Studies Using *MscI/BalI* Polymorphism*

Reference	Country/ethnicity	Sample size		Allele 1 frequency		Association
		Patients	Controls	Patients	Controls	
Crocq et al. [1992]	UK/C	68	68	.68 ± .03	.62 ± .03	+[a]
	France/C	73	71	.68 ± .04	.65 ± .04	+[a]
Mant et al. [1994]	UK/C	134	166	.67 ± .07	.62 ± .07	+[a]
Laurent et al. [1994]	France/C	76	86	.68 ± .04	.66 ± .04	−
Jonsson et al. [1993]	Sweden/C	76	53	.68 ± .04	.72 ± .04	−[b]
Nothen et al. [1993]	Germany/C	111	100	.72 ± .03	.70 ± .03	−
DiBella et al. [1993]	Italy/C	85	78	.68 ± .04	.58 ± .04	−
	Italy/C	52	78	.75 ± .04	.58 ± .04	+[c]
Yang et al. [1993]	China/Ch	107	98	.71 ± .03	.71 ± .03	−
Nanko et al. [1993]	Japan/J	91	90	.72 ± .03	.72 ± .03	−
Saha et al. [1995]	Singapore/Ch	137	125	.69 ± .03	.70 ± .03	−
Nimgaonkar et al. [1993]	USA/C	53	61	.71 ± .04	.62 ± .04	+[d]

*C, Caucasian; Ch, Chinese; J, Japanese.
[a]With homozygosity.
[b]Between homozygosity and responsiveness to neuroleptics (uncorrected for multiple comparisons).
[c]Between allele 1 and delusional disorder.
[d]Between allele 1 and family history of positive patients.

Recent studies involving multigenerational pedigrees have not detected linkage between schizophrenia and the D3RG locus, calling into question the relevance of the association detected in the above studies [Wiese et al., 1993; Sabate et al., 1994]. If D3RG does not contribute substantially to schizophrenia susceptibility (as suggested by the relatively low odds ratios in the reported studies), linkage may not be detectable. In addition, linkage may not be detectable if the mode of inheritance is misspecified or genetic heterogeneity is present. If an association is indeed present, the assumptions of linkage analysis may be confounded [Hodge and Spence, 1983]. Finally, distinction has been made between a "necessary disease allele" and a "susceptibility allele" in the etiology of genetically complex diseases [Greenberg and Hodge, 1989; Greenberg, 1993; Hodge, 1993]. While the former *has* to be present for disease expression, the latter is neither necessary nor sufficient for disease manifestation. Association could be detectable in the presence of both types of alleles, but linkage would only be detectable in the presence of "necessary alleles" [Hodge, 1993]. Under this classification, D3RG would be classified as a "susceptibility" locus and significant linkage would not necessarily be expected.

In summary, despite the large number of studies, a satisfactory consensus has not been attained about the etiological role of D3RG has not been attained. It is tempting to dismiss the original findings because of the discrepancies listed above. However, in view of the methodological problems among the negative studies, a thorough assessment of the putative association is warranted.

In the present study, pooled data from published association studies were initially analyzed. In addition, the study was designed to overcome some of the deficiencies of the earlier reports. The study sample was enlarged from our previous report [Nimgaonkar et al., 1993] to include individuals of Caucasian and African-American ethnicity. Two groups of controls were used for comparison. The first consisted of adults matched for ethnicity, who were screened to have no history of substance abuse or major psychiatric illness. The second control group included umbilical cord blood samples from live births at a local hospital. The latter provided estimates of the "true" population frequencies of D3RG alleles. In view of the possible associations with subgroups reviewed above, multivariate analysis was performed after pooling the results from the patients and controls of both ethnic groups. Individual and joint effects of D3RG genotype, ethnicity, and gender in predicting illness status were thus investigated. In view of the discrepant results, an association study was also conducted using an independently sampled U.S. cohort, which was used earlier to demonstrate an association with the porphobilinogen deaminase gene locus [Sanders et al., 1992].

MATERIALS AND METHODS

Clinical

Pittsburgh cohort. The cohort included inpatients and outpatients being treated at the Schizophrenia Treatment and Research Center, Western Psychiatric Institute and Clinic (WPIC). WPIC is a tertiary care facility, and also a catchment area Community Mental Health Center for a geographically defined area in Allegheny County, western Pennsylvania. Patients of Caucasian and African-American ethnicity with a diagnosis of schizophrenia by DSM-III-R criteria were recruited into the study. The Caucasian individuals included those reported on earlier [Nimgaonkar et al., 1993]. A semistructured interview based on the Schedule for Affective Disorders and Schizophrenia (SADS), [Spitzer et al., 1978] was administered to each patient, in order to elicit psychopathology. Age of onset was defined as the year in which medical help was sought for psychiatric abnormalities or the age at which such abnormalities first caused subjective distress or impaired function. Family history of psychiatric illness in first- and second-degree relatives was obtained from medical records and by interviewing each patient. Each patient's response to pharmacotherapy during an inpatient stay was rated as full, partial, or poor resolution of delusions, hallucinations, or thought disorder. This information, along with the information obtained from medical records, was entered into OPCRIT 3.3, a computerized diagnostic symptom checklist [McGuffin et al., 1991].

The adult controls were screened for a lifetime history of substance abuse, psychosis, or major depressive disorder (Research Diagnostic Criteria: RDC) [Spitzer et al., 1978]. They were matched for ethnicity to the patients and resided in the same geographic area. Thus, both groups had similar socioeconomic backgrounds. Family history of psychiatric illness was obtained from each participant. Venous blood (20 ml) was drawn from each individual after written informed consent was obtained.

The second group of controls consisted of neonates born during 1993–1994 at Magee-Women's Hospital (MWH) in Pittsburgh, a local hospital serving a catchment area overlapping that of WPIC. Umbilical cord blood samples (10 ml) were obtained from live births of Caucasian and African-American ethnicity.

Houston cohort. The sample consisted of unrelated individuals with schizophrenia (n = 28) or schizoaffective disorder (n = 22) diagnosed by DSM-III-R criteria. The latter fulfilled RDC for schizoaffective disorder, mainly schizophrenic. They were predominantly drawn from the Veteran Affairs Medical Center (VAMC) Psychiatry Service, Houston, Texas. Family history of psychiatric illness in first-degree relatives was obtained from medical records and by interviewing each patient. Familiality with respect to schizophrenia was defined using Family History RDC [Andreasen et al., 1986]. The clinical information was stored using OPCRIT 3.3. The controls were adults attending the Medical Service at the same facility. Both groups were of Northern European descent. Further information about the cohort has been published [Sanders et al., 1992].

Molecular Genetic Analysis

Lymphocytes were extracted from venous blood using density gradient centrifugation, and genomic DNA was

extracted by the high salt method [Miller et al., 1988]. DNA was extracted directly from the cord blood sample using a commercial kit (QIAmp Kits, QIAGEN Inc., Chatsworth, CA). Genomic DNA was subjected to polymerase chain reaction (PCR) to amplify the first exon of D3RG, using a published method [Crocq et al., 1992; Lannfelt et al., 1992; Nimgaonkar et al., 1993]. Briefly, genomic DNA (200 ng) was amplified with PCR buffer (Gibco BRL, Gaithersburg, MD), $MgCl_2$ (2 mM), dNTPS (50 μM each), *Taq* polymerase (1.25 units, Boehringer Mannheim, Indianapolis, IN), and primers (200 nM each) in a total volume of 50 μl. The forward and reverse primer sequences used were 5′-GCTCTATCTC-CAACTCTCACA-3′ and 5′-AAGTCTACTCACCTC-CAGGTA-3′, respectively. PCR involved initial denaturation of 94°C for 7 min, followed by 30 cycles of annealing at 55°C for 30 sec, polymerization at 72°C for 30 sec, and denaturation at 94°C for 90 sec, with a final polymerization at 72°C for 10 min. Following PCR, the amplified DNA was digested with the restriction endonuclease *Msc*I, an isoschizomer of *Bal*I (3U, New England Biolabs, Beverly, MA), in a total volume of 25 μl. The digested fragments were electrophoresed in 2% agarose, and visualized using ethidium bromide stain. The size of the fragments was estimated using molecular weight markers, and genotypes were obtained for each individual as described in Crocq et al. [1992].

Statistical Analysis

The pooled results from the published studies were analyzed using Woolf's method. Student's t-test and the chi-square test were used to compare patients and controls, as appropriate. When cell sizes were small, the likelihood ratio or Fisher's chi-square tests were used. Multivariate analysis was performed using the computer program Statistical Program for Social Sciences (SPSS, Chicago, Ill). Life tables were plotted for the patients using age of onset as the outcome variable. Survival curves were compared using Kaplan-Meier analysis (Breslow test).

RESULTS

Analysis of Published Results

The results of the studies listed in Table I were pooled and analyzed using Woolf's method. When allele frequencies among patients (n = 995) and controls (n = 1,006) were compared, a nonsignificant excess of allele 1 was noted among the former (estimated mean odds ratio 1.12, pooled $\chi^2 = 2.45$, 1 *df*). There was no significant evidence for heterogeneity ($\chi^2 = 5.29$, 9 *df*).

The pooled results were also dichotomized into homozygotes and heterozygotes, as suggested by Crocq et al. [1992]. There was a significant excess of homozygotes among the patients (estimated mean odds ratio 1.21, 95% C.I. 1.03, 1.39, pooled $\chi^2 = 4.15$, $P < 0.05$, 1 *df*). There was no significant evidence for heterogeneity ($\chi^2 = 14.78$, 9 *df*).

Associations Between D3RG and Schizoprenia in the Pittsburgh Sample

Demographic features of the patients and adult controls are given in Table II. There was no significant difference in gender distribution in either ethnic group or in the whole sample, but patients were significantly older than controls in each ethnic group. Information about gender distribution in the neonatal sample was not available.

Associations between schizophrenia and the D3RG locus were next tested. The distributions of D3RG genotypes and D3RG allele frequencies were used for comparison. The frequency of allele 1 among Caucasians is almost double that among African-Americans (Table II). In view of this difference, the two ethnic groups were initially analyzed separately.

African-American cohort. Genotype distributions for patients and both groups of controls were in Hardy-Weinberg equilibrium. There was a significant difference in the distribution of genotypes between the patients and adult controls (Table II, $\chi^2 = 8.0$, $P < 0.02$, 2 *df*). The difference was restricted to female patients (results not shown). In contrast, no significant differences in distribution of genotypes among patients and neonates were observed. There was no significant difference in allelic distribution between patients and either group of controls. A significant excess of 1/1 and 2/2 homozygotes was not observed among patients, though a significant excess of allele 1 homozygotes was observed in comparison with adult controls only ($\chi^2 = 7.3$, $P < 0.007$, 1 *df*).

Caucasian cohort. No significant deviations from Hardy-Weinberg expectations were noted among pa-

TABLE II. Demographic Characteristics and D3 Receptor Genotypes (Pittsburgh Sample)†

						*Msc*I genotypes			
Ethnicity	Group	n	Age	Male	Female	1/1	1/2	2/2	Allele 1 frequency
African-American	Patients[a]	65	37.2 ± 9.9*	29	36	13	22	30	.37 ± .04
	Adult controls[b]	63	31.6 ± 9.8	27	36	3	30	30	.29 ± .04
	Neonates	60		?	?	9	22	29	.34 ± .04
Caucasian	Patients	65	37.2 ± 10.8*	39	26	33	26	6	.71 ± .04
	Adult controls	65	31.1 ± 10.2	31	34	27	30	8	.65 ± .04
	Neonates	100		?	?	44	45	11	.68 ± .03

†Genotypes are in accordance with published nomenclature [Crocq et al., 1992].
[a]Genotype distribution among African-American patients vs. adult controls, $\chi^2 = 8.0$, $P < 0.02$, 2 *df*.
[b]Genotype distribution among African-American adult controls vs. Caucasian adult controls, $\chi^2 = 35.6$, $P < 0.0001$, 2 *df*. Age presented as mean ± SD.
*Significantly different from controls, $P < 0.001$, Student's t-test.

tients or controls. Patients did not differ significantly from adult or neonatal controls with respect to genotype distribution (Table II). No gender-specific differences were noted. An excess of homozygotes was not observed among patients.

Combined cohort. The above analyses suggest that ethnicity and gender may influence allele frequencies at the D3RG locus. Multivariate analysis was therefore performed. Using illness status as the outcome variable, logistic regression with backward elimination was used to examine the individual and joint effects of D3RG genotype, ethnicity, and gender. Only D3RG allele 1 homozygotes significantly predicted illness status (β coefficient = 4.1, $P < 005$, 1 *df*, 46/130 patients were homozygous, vs. 30/130 among controls; $\chi^2 = 4.76$, $P < 0.03$). All the variables, apart from allele 1 homozygosity, were sequentially removed from the regression equation, without significantly altering goodness of fit. D3RG allele 1 homozygosity thus remained the only significant predictor of illness. Interactions between different combinations of variables were also tested, using log linear analysis. The only significant model involved illness status and D3RG status (results not shown).

Logistic regression analysis was also conducted among patients and neonatal controls, and the effects of ethnicity and D3RG genotype were examined. Ethnicity, but not D3RG genotype, had a significant contribution (results not shown).

Association of D3RG With Subgroups of Patients

The above results suggest a relatively small contribution of D3RG allele 1 to illness susceptibility (odds ratios: Caucasians, 1.33; African-Americans, 1.46). It is possible that more significant associations exist among subgroups of patients. Therefore, associations using the following variables were examined: familiality, age of onset, clinical response to antipsychotic drugs, and psychopathological features.

Familiality. Patients were subdivided on the basis of family history of schizophrenia (first- or second-degree relatives). A more restrictive definition (positive family history among first-degree relatives only) was also used. None of the adult controls had a family history of schizophrenia, but information was unavailable for some individuals (Caucasians, n = 4; African-Americans, n = 14).

African-American cohort. Adequate family histories were unavailable for 19 patients. A significant difference in genotype distribution was observed between family history-positive patients and adult controls, when familiality was defined as a history of schizophrenia in first- or second-degree relatives ($\chi^2 = 6.5$, $P < 0.04$, 2 *df*, Table III). Interestingly, patients without such a family history also had a significantly different genotype distribution compared with adult controls ($\chi^2 = 6.7$, $P < 0.04$, 2 *df*). Using the more restrictive definition, genotype distribution among family history-positive patients was not different from that of adult controls ($\chi^2 = 3.8$, 2 *df*, n.s., Table III). On the other hand, there was a significant difference among patients without such a family history ($\chi^2 = 9.1$, 2 *df*, $P < 0.02$). There were no significant differences in genotype distributions among patients with and without a family history, using either definition of familiality. In view of the substantial number of patients for whom a family history was unavailable, the genotype distribution of this group was compared with controls and with the other patients. There were no significant differences. When neonatal controls were used for comparison instead of adults, no significant differences between family history-positive patients and controls emerged. All these comparisons were repeated using allele frequencies. No significant differences were noted, though there was a nonsignificant trend for an increased frequency of allele 1 among all subgroups of patients.

These results suggest that among African-Americans, genotype distributions are different when patients and adult controls are compared. However, such differences are not dependent on subdivision based on familiality.

Caucasian cohort. A reliable family history could not be obtained for some patients (n = 7). As reported earlier in a smaller sample [Nimgaonkar et al., 1993], the distribution of genotypes among patients with a

TABLE III. *Msc*I Genotypes of Patients Based on Family History of Schizophrenia (Pittsburgh Sample)*

Ethnicity	Family history of schizophrenia	n	*Msc*I genotypes 1/1	1/2	2/2	Allele 1 Frequency	Comparison group	χ^2	*P*
African-American	1° or 2° relatives	20	5	6	9	.40 ± .07	All adult controls	6.5	<.04
	1° relatives	13	3	5	5	.42 ± .10	All adult controls	3.8	n.s.
	Absent (1° or 2°)	26	6	8	12	.38 ± .07	All adult controls	6.7	<.04
	Absent (1°)	33	8	9	16	.38 ± .06	All adult controls	9.1	<.02
	Unknown	19	2	8	9	.32 ± .08	All adult controls	0.8	n.s.
Caucasian	1° or 2° relatives	13	12	1	0	.98 ± .03	All adult controls	13.3	<.002
							Neonatal controls	12.8	<.002
	1° relatives	10	9	1	0	.95 ± .05	All adult controls	9.6	<.01
							Neonatal controls	9.1	<.01
	Absent (1° or 2°)	45	20	19	6	.66 ± .05	Familial patients (1° or 2°)	11.4	<.004
	Absent (1°)	48	23	19	6	.68 ± .05	Familial patients (1°)	7.4	<.003
	Unknown	7	1	6	0	.57 ± .13	All adult controls	4.8	n.s.

*Genotypes are in accordance with published nomenclature [Crocq et al., 1992; Nimgaonkar et al., 1993]. Genotype distributions were compared using the likelihood ratio chi-square test (2 *df*). Genotype distributions for controls are listed in Table II. n.s., not significant.

history of schizophrenia among first- or second-degree relatives differed significantly from adult controls (χ^2 = 13.3, $P < 0.002$, Table III). An increased frequency of allele 1 was also observed (χ^2 = 13.5, $P < 0.0002$; odds ratio 13.69, C.I. 1.80, 104.30). Significant differences in the distribution of genotypes and alleles were also noted when family history-positive patients were compared with neonatal controls (genotype distribution, χ^2 = 12.8, $P < 0.002$, 2 *df*; allele frequencies, χ^2 = 12.9, $P < 0.0003$, 1 *df*).

Similarly, patients with a first-degree relative diagnosed to have schizophrenia had a significantly different genotype distribution in comparison with either adult controls (χ^2 = 9.6, $P < 0.01$) or neonatal controls (χ^2 = 9.1, $P < 0.02$). An excess of allele 1 was also present among such patients in comparison with either group of controls (adult controls, χ^2 = 9.6, $P < 0.002$; neonatal controls, χ^2 = 9.1, $P < 0.003$). Using either definition of familiality, a significant difference in genotype distribution was observed between patients with and without a family history of schizophrenia (Table III). Unlike the African-American patients, no significant differences in genotype distributions between adult controls and family history-negative patients were noted.

These results support an association between D3RG allele 1 and Caucasian family history-positive patients.

Age of onset (AOO) and MscI genotype. Using the Kaplan-Meier method, comparison of survival curves with AOO as outcome revealed significant differences between male and female patients (median AOO: male patients, 19.0 years; female patients, 22.0 years; Breslow statistic = 6.13, 1 *df*, $P < 0.02$). No significant differences were noted when patients were subdivided by ethnicity or family history (results not shown). Therefore, survival curves for patients with the three *Msc*I genotypes were compared following stratification by gender. Overall, a significant difference was noted (Breslow statistic = 8.64, 2 *df*, $P < 0.02$). Further pairwise comparisons between genotypes among male and female patients revealed that the only significant differences occurred among males: survival curves for allele 2 homozygous individuals were significantly different from heterozygotes (Breslow statistic = 5.03, $P < 0.03$), as well as allele 1 homozygotes (Breslow statistic = 9.73, $P < 0.002$). Thus, allele 2 homozygosity may be associated with early AOO (Fig. 1).

Psychopathology. It is possible that associations with individual abnormalities exist. Therefore, the OPCRIT checklist was used to identify lifetime presence of the following abnormalities among patients: bizarre behavior, blunted affect, inappropriate affect, hallucinations in any modality, auditory hallucinations, nonauditory hallucinations, positive thought disorder, negative thought disorder, paranoid delusions, and nonparanoid delusions and insight. Presence or absence of syndromal clusters was also analyzed. These included first-rank symptoms, delusions with hallucinations, and paranoid delusions with hallucinations. No consistent associations were noted when the two ethnic groups were analyzed separately. Appropriate corrections for multiple comparisons were made in these analyses (data not shown).

Clinical response to drugs. No significant differences in genotype distributions were noted when patients subdivided on the basis of response to pharmacotherapy were compared with controls, or when comparisons between subgroups were made (data not shown).

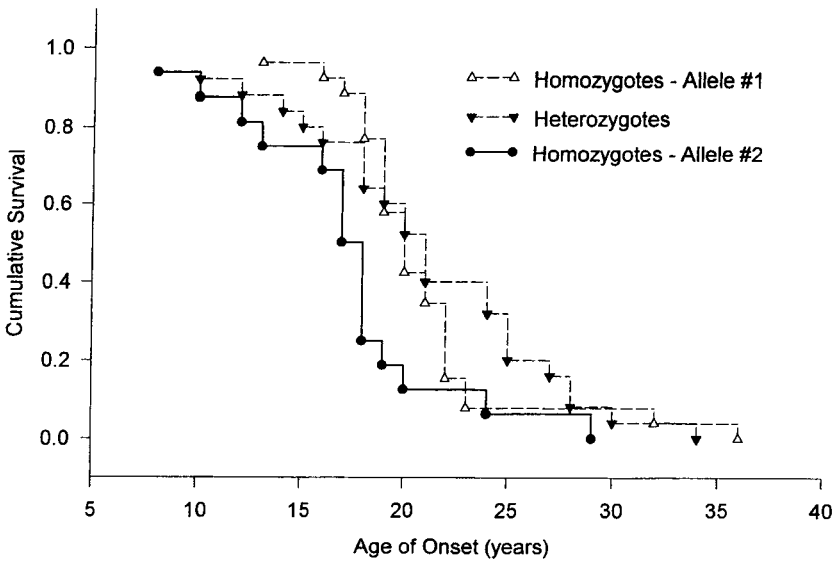

Fig. 1. Age of onset (AOO) was plotted as a survival function of dopamine D3 receptor genotype among male patients with schizophrenia (n = 68, Pittsburgh sample). AOO was defined as age in years in which medical help was sought for psychiatric abnormalities, or the age at which such abnormalities first caused subjective distress or impaired function. Using the Kaplan-Meier method, survival curves for allele 2 homozygous individuals were significantly different from heterozygous, as well as allele 1 homozygous, individuals.

Associations Between D3RG and Schizophrenia in the Houston Sample

There were 4 female patients and 3 female controls. Thus, there were no significant differences in gender distribution between patients and controls. However, the patients were significantly younger (patients, 45.6 ± 14.0 years; controls, 61.6 ± 10.0 years; mean ± SD; $P < 0.0001$, Student's t-test).

Genotype distributions among controls and patients are shown in Table IV. No significant differences in genotype distribution or allele frequencies were noted between the two groups. Patients were also subdivided on the basis of diagnosis (schizophrenia or schizoaffective disorder, mainly schizophrenic), family history of schizophrenia (first degree relatives), or presence of substance abuse. No significant differences in genotype distribution or allele frequencies were noted in comparison with controls or within subgroups.

Due to the small number of female patients (n = 4) and the possibility that AOO is later among women [Angermeyer and Kuhn, 1988], AOO was examined only among male patients. Comparison of survival curves with AOO as outcome did not reveal significant differences between patients grouped on the basis of the *Msc*I genotype (Breslow statistic = 0.22, 2 *df*).

Thus, no significant evidence for an association with the D3RG locus could be detected, unlike the Pittsburgh sample.

Comparison of Pittsburgh and Houston Cohorts

In order to identify possible reasons for the disparity between the Pittsburgh and Houston samples, individuals of Caucasian ethnicity from the two cohorts were compared. The patients from Pittsburgh included more women ($P < 0.0002$, likelihood ratio $\chi^2 = 14.5$, 1 *df*), as did the Pittsburgh controls ($P < 0.00001$, likelihood ratio $\chi^2 = 32.5$, 1 *df*). The mean age of onset among the Pittsburgh patients was significantly earlier than the Houston patients, even when gender was used as a covariate (Pittsburgh, 20.8 ± 5.7 years; Houston, 25.8 ± 9.5 years; mean ± SD; $P < 0.001$, $F = 14.23$, 1 *df*, analysis of covariance with gender as covariate). The proportion of patients with a positive family history among first-degree relatives was similar in the two groups.

TABLE IV. Characteristics of Houston Sample

		Genotype		
Group	n	1/1	1/2	2/2
Controls	51	27	17	7
All patients	50	24	22	4
Subgroups of patients				
Schizophrenia	28	12	14	2
Schizoaffective psychosis	22	12	8	2
Family history[a]				
Present	6	3	3	0
Absent	44	21	19	4
Substance abuse				
Present	23	12	10	1
Absent	27	12	12	3

[a]Family history of schizophrenia (first-degree relatives).

DISCUSSION

Analysis of pooled results from published studies suggested an excess of homozygotes among patients, but no significant association with allele 1. Such analysis may be criticized due to variations in criteria for selection of controls in the different studies, as well as possible differences in clinical characteristics of the patients. These possibilities were addressed in the present study. The strengths of this investigation stem from two independently sampled cohorts. The cohorts included a moderately large sample of patients belonging to two ethnic groups, as well as two types of ethnically matched controls. Analysis of D3 genotypes in the Pittsburgh cohort did not reveal an excess of homozygotes among either group of patients, as suggested earlier [Crocq et al., 1992]. On the other hand, multivariate analysis supported a significant contribution of D3RG allele 1 homozygous status to schizophrenia. The overall increase in risk appeared to be small. Furthermore, the increased risk was not evident when neonatal controls were used in the analysis. In contrast, analysis of the Texas cohort did not suggest a significant association with either the illness or its age of onset.

Among the Pittsburgh patients, the differing results obtained with the two control groups are of interest, and may explain some of the discrepancies in the earlier reports (Table I). There are important differences between the neonatal and the adult control samples, though both groups were drawn from the same geographical area. While the neonates were unselected, the adults were volunteers who were carefully screened for absence of substance abuse. During the course of a genetic study of alcoholism, Gelernter et al. [1991] suggested that controls selected for the absence of alcoholism need not obscure true associations at candidate gene loci, especially if the risk contributed by an allele is large. In the present study, controls were screened not only for absence of psychotic illnesses, but also for absence of substance abuse and of major depressive disorders. Since the number and genotypic distribution of controls rejected from the study is not known, it is difficult to say if the significant differences between patients and controls resulted from the screening process. Nevertheless, a systematic bias in selection of adult (volunteer) controls cannot be ruled out. Interestingly, the screening process appears to have also removed controls with a family history of schizophrenia. Ascertainment bias may be reduced using the haplotype relative risk method [Falk and Rubinstein, 1987]. One such study supports an association of schizophrenia with homozygosity at D3RG [Owen, personal communication].

The strong association of allele 1 with familiality among individuals of Caucasian ethnicity remained even after the cohort reported on earlier [Nimgaonkar et al., 1993] was enlarged. It was not dependent on type of control group used for comparison. An association among a familial subgroup would be predicted if inheritance of schizophrenia is polygenic/multifactorial, and possession of allele 1 increases the risk for schizophrenia. Analysis of family history-positive patients would

enrich the sample for individuals with a greater genetic predisposition, and consequently increase the strength of an association with D3RG [Murray et al., 1985]. Other groups have failed to detect associations with family history-positive patients [Jonsson et al.,1993; Nothen et al., 1993; Nanko et al., 1993; DiBella et al., 1993; Sabate et al., 1994]. The discrepancy could be due to inherent inaccuracies in the use of the family history method for ascertaining individuals with an increased genetic predisposition [Eaves et al., 1986]. It could also reflect differences in predisposing genetic factors among the various populations investigated (Table I).

In contrast with the Caucasians, the findings among the family history-positive African-American patients were variable. Differences in genotype distributions between familial patients and adult controls were significant only when familiality was defined on the basis of illness in first- or second-degree relatives. Surprisingly, significant differences were also evident when nonfamilial patients were compared with adult controls. Using neonatal controls for comparison eliminated such differences. The variability may be due to the large number of patients for whom family history information was unavailable. It is also possible that a "true" association with the D3RG locus does not exist among African-Americans, and that the variable findings result from admixture with Caucasians. Significant admixture between the two ethnic groups has been shown [Chakraborty et al., 1992]. Notably, the frequency of allele 1 among controls in the Congo is 0.09 [Crocq, personal communication]. The allele frequencies noted among African-American individuals in the present study are compatible with the admixture proposed by Chakraborty et al. [1992].

A genetic contribution to age of onset of schizophrenia is presumed, because of significant correlations in AOO among family members and affected siblings, as well as twins [reviewed by Kendler et al., 1987]. Furthermore, it is thought that factors influencing AOO are different from those influencing liability to schizophrenia [Kendler and Maclean, 1990]. The onset of schizophrenia is generally delayed in women [Angermeyer and Kuhn, 1988]. In agreement with previous reports, the male patients in the present study had earlier AOO than the female patients. Survival analysis suggests an earlier AOO for male patients homozygous for allele 2. Similar differences have been noted among patients in Cardiff, U.K. and Rouffach, France [Owen and Crocq, personal communication]. These results are intriguing: if allele 1 homozygosity increases liability to schizophrenia, it would also be expected to lower the AOO.

Alternatively, the presence of the allele 2 homozygous state may independently lower the AOO. Such complex effects on liability as well as AOO may lead to subtle differences in genotype distribution among samples of patients with different ages of onset. For example, patients with relatively early AOO are more likely to be homozygous for allele 2, if the present findings are replicable. Such differences may explain the variable associations summarized in Table I.

To our knowledge, three other groups have examined the relationship between AOO and the *Msc*I polymorphisms at the D3RG locus [Jonsson et al., 1993; Nanko et al., 1993; Nothen et al., 1993]. No significant associations were noted. While one study did not specify the method of analysis [Nothen et al., 1993], the other two examined allele frequencies among patients subdivided on the basis of arbitrarily selected AOO. Other variables known to influence AOO, such as gender, were presumably not included in these analyses.

In contrast to the results from the Pittsburgh sample, the Houston study did not yield significant evidence for the putative association between schizophrenia and the D3RG locus, either with respect to an excess of homozygosity [Crocq et al., 1992], or with respect to allele 1 frequencies. There are several possible reasons for these discrepancies. The most likely reason is a difference in sample size. It was estimated earlier that a sample size of approximately 100 (patients + controls) would have a power of 0.8 to detect an effect of the magnitude reported by Crocq et al. [1992], with α set at 0.05 (one-tailed comparison) [Nimgaonkar et al., 1993]. Since the association with allele 1 reported by Nimgaonkar et al. [1993] is of a smaller magnitude, the required sample size would be even larger. Thus, the possibility of a false-negative result from the present sample cannot be ignored.

The discrepancies may also be attributable to differences in the controls in the two studies. Laurent et al. [1994], who conducted an association study in France, did not detect an association with homozygosity. They attributed the negative results to differences in allele frequencies among their controls, in comparison with an earlier French study [Crocq et al., 1992]. Our studies also suggest an important role for the controls. As discussed above, the association with allele 1 among the Pittsburgh patients was noted when screened adult controls were used for comparison, but not when unscreened neonatal controls were used. The frequency of allele 1 was higher among the Houston controls, in comparison with the adult Caucasian controls in the Pittsburgh sample, but the difference was not statistically significant. Therefore, this difference in itself may not account for the discrepant results.

The Pittsburgh and Houston cohorts also differed significantly in gender distribution, with relatively more males among the latter. Notably, most studies published to date have not revealed gender-related differences in allele frequencies. Indeed, Mant et al. [1993] reported that the homozygote excess was more marked among male patients. Therefore, the present sample would be more suited to detect such an effect. Moreover, the frequency of allele 1 among the Houston patients was almost identical to the frequency among the Pittsburgh patients (Tables II and III). Thus, gender differences cannot account for the differing results.

In contrast, allele 1 appeared to be more frequent among the familial patients at Pittsburgh, in comparison with the familial patients at Houston. A smaller proportion of the Houston patients also had a positive family history for schizophrenia, compared with the Pittsburgh cohort (0.14 vs. 0.21). Though neither of these differences attained statistical significance, the trends may have contributed to the overall negative re-

sults in the Houston cohort. It is of interest that the significant association with homozygosity in the study by Mant et al. [1994] was most marked among patients with a family history of schizophrenia. Thus, the familial patients may contribute substantially to the overall association, and differences between cohorts with respect to the proportion of family history-positive patients may determine whether an association is detected.

Finally, analysis of the Pittsburgh cohort suggests that the D3RG locus, or a linked locus, may not only be associated with the liability to schizophrenia, but may also influence AOO. In this context, it is notable that the Houston patients had a significantly later AOO compared with the Pittsburgh patients. This difference may also have contributed to the different results from the two studies.

In conclusion, multiple regression analysis suggests that allele 1 homozygosity at the D3RG locus increases the risk for schizophrenia. This locus, or a linked locus, may also influence the age of onset of schizophrenia. The predisposition to schizophrenia explained by allelic status at D3RG is small. Furthermore, it is different even among Caucasians and African-Americans. Such variability is compatible with a polygenic/multifactorial mode of inheritance for schizophrenia [Gottesman and Shields, 1967]. If other loci contribute risks of a similar magnitude, large-scale association studies will be required in order to dissect the complex etiology of schizophrenia. These findings are in disagreement with the negative results from the Houston sample. The different results may be due to a number of causes, including smaller sample size, differences in the prevalence of familiality, or age of onset among patients. They could also be attributed to differences in the selection of controls, a possibility which could also account for the discrepancies in prior studies. The results also illustrate potential pitfalls in association studies.

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REFERENCES

Andreasen NC, Rice J, Endicott J, Reich T, Coryell W (1986): The family history approach to diagnosis: How useful is it? Arch Gen Psychiatry 43:421–429.

Angermeyer MC, Kuhn L (1988): Gender differences in age at onset of schizophrenia. An overview. Eur Arch Psychiatry Neurol Sci 237: 351–364.

Beckman L, Frohlander N (1990): Heterozygosity effects in studies of genetic markers and disease. Hum Hered 40:322–329.

Bodmer WF (1987): The human genome sequence and the analysis of human traits. Ciba Found Symp 130:215–225.

Cagle M, Peacock ML, Mathay JG, Sunnenberg C, Tandon R, Meltzer HY, Fink JK (1993): Dopamine D3 receptor polymorphism in schizophrenia: Importance of evaluating control subjects. Am J Hum Genet 53:783.

Carter CL, Chung CS (1981): Segregation analysis of schizophrenia under a mixed genetic model. Hum Hered 31:325–333.

Chakraborty R, Kamboh MI, Nwankwo M, Ferrell RE (1992): Caucasian genes in American Blacks: New data. Am J Hum Genet 50:145–155.

Cooper DN, Clayton JF (1988): DNA polymorphism and the study of disease associations. Hum Genet 78:299–312.

Cox NJ, Bell GI (1989): Disease associations. Chance, artifact, or susceptibility genes? Diabetes 38:947–950.

Crocq MA, Mant R, Asherson P, Williams J, Hode Y, Mayerova A, Collier D, Lannfelt L, Sokoloff P, Schwartz JC, (1992): Association between schizophrenia and homozygosity at the dopamine D3 receptor gene. J Med Genet 29:858–860.

DiBella D, Catalano M, Nobile M, Novelli E, Noethen MM, Cichon S, Smeraldi E (1993): No association between homozygosity at the dopamine D3 receptor locus and psychotic disorders. Psychiatr Genet 3:42.

Eaves L, Kendler KS, Schulz SC (1986): The familial sporadic classification: Its power for the resolution of genetic and environmental etiological factors. J Psychiatr Res 20:115–130.

Falk CT, Rubinstein P (1987): Haplotype relative risks: An easy, reliable way to construct a proper control sample for risk calculations. Ann Hum Genet 51:227–233.

Gelernter J, O'Malley S, Risch N, Kranzler HR, Krystal J, Merikangas K, Kennedy JL, Kidd KK (1991). No association between an allele at the D2 dopamine receptor gene (DRD2) and alcoholism. JAMA, 266: 1801–1807.

Gottesman II, Shields J (1967): A polygenic theory of schizophrenia. Proc Natl Acad Sci USA 58:199–205.

Gottesman I, Shields J (1982): "Schizophrenia, the Epigenetic Puzzle." Cambridge: Cambridge University Press, pp. 209–233.

Greenberg DA (1993): Linkage analysis of "necessary" disease loci versus "susceptibility" loci. Am J Hum Genet 52:135–143.

Greenberg DA, Hodge SE (1989): Linkage analysis under "random" and "genetic" reduced penetrance. Genet Epidemiol 6:259–264.

Hodge SE (1993): Linkage analysis versus association analysis: Distinguishing between two models that explain disease-marker associations. Am J Hum Genet 53:367–384.

Hodge SE, Spence MA (1983): Some epistatic two-locus models of disease. II. The confounding of linkage and association. Am J Hum Genet 33:396–406.

Jonsson E, Lannfelt L, Sokoloff P, Schwartz JC, Sedvall G (1993): Lack of association between schizophrenia and alleles in the dopamine D3 receptor gene. Acta Psychiatr Scand 87:345–349.

Kendler KS, MacLean CJ (1990): Estimating familial effects on age at onset and liability to schizophrenia. I. Results of a large sample family study. Genet Epidemiol 7:409–417.

Kendler KS, Tsuang MT, Hays P (1987): Age at onset in schizophrenia. A familial perspective. Arch Gen Psychiatry 44:881–890.

Lannfelt L, Sokoloff P, Martres MP, Pilon C, Giros B, Schwartz JC, Jonsson E, Sedvall G (1992). Amino acid substitution in the dopamine D3 receptor as a useful polymorphism for investigating psychiatric disorders. Psychiatr Res 46:69–78.

Laurent C, Savoye C, Samolyk D, Meloni R, Mallet J, Campion D, Martinez M, d'Amato T, Bastard C, Dollfus S (1994): Homozygosity at the dopamine D3 receptor locus is not associated with schizophrenia. J Med Genet 31:260–264.

Mant R, Williams J, Asherson P, Parfitt E, McGuffin P, Owen MJ (1994): Relationship between homozygosity at the dopamine D3 receptor gene and schizophrenia. Am J Med Genet 54:21–26.

McGue M, Gottesman II, Rao DC (1983): The transmission of schizophrenia under a multifactorial threshold model. Am J Hum Genet 35:1161–1178.

McGuffin P, Farmer AE, Harvey I (1991): A polydiagnostic application of operational criteria in studies of psychotic illness. Arch Gen Psychiatry 48:764–770.

Miller SA, Dykes DD, Polesky HF (1988): A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Morell R (1993): Association between schizophrenia and homozygosity at the dopamine D3 receptor gene. J Med Genet 30:708.

Murray RM, Lewis SW, Reveley AM (1985): Towards an etiological classification of schizophrenia. Lancet 1:1023–1026.

Nanko S, Sasaki T, Fukuda R, Hattori M, Dai XY, Kazamatsuri H, Kuwata S, Juji T, Gill M (1993): A study of the association between schizophrenia and the dopamine D3 receptor gene. Hum Genet 92:336–338.

Nimgaonkar VL, Zhang XR, Caldwell JG, Ganguli R, Chakravarti, A (1993): Association study of schizophrenia with dopamine D3 receptor gene polymorphisms: Probable effects of family history of schizophrenia. Am J Med Genet (Neuropsychiatric Genet) 48: 214–217.

Nothen MM, Korner J, Lannfelt L, Sokoloff P, Schwartz JC, Lanczik M, Rietschel M, Cichon S, Kramer R, Fimmers R, Moller HJ, Beckmann H, Propping P, Grandy DK, Civelli O, O'Dowd BF (1993): Lack of association between schizophrenia and alleles of the dopamine D1, D2, D3 and D4 receptor loci. Psychiatr Genet 3:89–94.

Owen MJ, Williams J, Mant R, (1993): Association between schizophrenia and homozygosity at the dopamine D3 receptor gene. J Med Genet 30:708–712.

Rao DC, Morton NE, Gottesman II, Lew R (1981): Path analysis of qualitative data on pairs of relatives: Application to. Am J Hum Genet 33:396–406.

Rietschel M, Nothen MM, Lannfelt L, Sokoloff P, Schwartz JC, Lanczik M, Fritze J, Cichon S, Fimmers R, Korner J, (1993): A serine to glycine substitution at position 9 in the extracellular N-terminal part of the dopamine D3 receptor protein: No role in the genetic predisposition to bipolar affective disorder. Psychiatry Res 46: 253–259.

Ritsner M, Sherina O, Ginath Y (1992): Genetic epidemiological study of schizophrenia: Reproduction behavior. Acta Psychiatr Scand 85:423–429.

Sabate O, Campion D, d'Amato T, Martres MP, Sokoloff P, Giros B, Leboyer M, Jay M, Guedj F, Thibaut F, (1994): Failure to find evidence for linkage or association between the dopamine D3 receptor gene and schizophrenia. Am J Psychiatry 151:107–111.

Saha N, Tsoi WF, Low PS, Basair J, Tay JSH (1995): Lack of association of the dopamine D3 receptor gene polymorphism (*BalI*) in Chinese schizophrenic males. Psychiatr Genet 4:201–204.

Sanders AR, Rincon-Limas DE, Chakraborty R, Grandchamp B, Hamilton JB, Fann WE, Patel PI (1992): Association between genetic variation at the porphobilinogen deaminase gene and schizophrenia. Schizophr Res 8:211–221.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990): Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146–151.

Spitzer RL, Endicott J, Robins E (1978): "Research Diagnostic Criteria (RDC) for a Selected Group of Functional Disorders," 3rd ed. New York: New York State Psychiatric Institute.

Wiese C, Lannfelt L, Kristbjarnarson H, Yang L, Zoega T, Sokoloff P, Ivarsson O, Schwartz JC, Moises HW, Helgason T (1993): No evidence of linkage between schizophrenia and D3 dopamine receptor gene locus in Icelandic pedigrees. Psychiatry Res 46:69–78.

Yang L, Li T, Wiese C, (1993): No association between schizophrenia and homozygosity at the D3 dopamine receptor gene. Am J Med Genet (Neuropsychiatric Genet) 48:83–86.