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Schizophrenia susceptibility associated with interstitial deletions of chromosome 22q11

(velocardiofacial syndrome/human/somatic cell hybrids/psychosis)

MARIA KARAYIORGOU*†, MICHAEL A. MORRIS‡, BERNICE MORROW§, ROBERT J. SHPRINTZEN¶, ROSALIE GOLDBERG§¶, JULIAN BORROW‖, ARNAUD GOS‡, GERALD NESTADT**, PAULA S. WOLYNIEC**, VIRGINIA K. LASSETER**, HARVEY EISEN*, BARTON CHILDS**, HAIG H. KAZAZIAN††, RAJU KUCHERLAPATI§, STYLIANOS E. ANTONARAKIS‡, ANN E. PULVER**, AND DAVID E. HOUSMAN‖

*Fred Hutchinson Cancer Research Center, Seattle, WA 98104; ‡Medical School and Division of Medical Genetics, Geneva University, Cantonal Hospital of Geneva, Switzerland 1211; §Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461; ¶Center for Craniofacial Disorders, Montefiore Medical Center, Bronx, NY 10467; ‖Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; **Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21231; and ††University of Pennsylvania Medical Center, Philadelphia, PA 19104

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ABSTRACT **We report the results of two studies examining the genetic overlap between schizophrenia and velocardiofacial syndrome. In study A, we characterize two interstitial deletions identified on chromosome 22q11 in a sample of schizophrenic patients. The size of the deletions was estimated to be between 1.5 and 2 megabases. In study B, we examine whether variations in deletion size are associated with the schizophrenic phenotype in velocardiofacial syndrome patients. Our results show that a region of the genome that has been previously implicated by genetic linkage analysis can harbor genetic lesions that increase the susceptibility to schizophrenia. Our findings should facilitate identification and cloning of the schizophrenia susceptibility gene(s) in this region and identification of more homogeneous subgroups of patients.**

Schizophrenia is a severe and disabling mental illness of variable expression and course, with 1% prevalence. Although the etiology of schizophrenia is unknown, data from family, twin, and adoption studies support the conclusion that both genes and experiences play some role in determining susceptibility. Results from segregation analyses have not been consistent in identifying a single mode of inheritance. As in most complex diseases, heterogeneity of cause is suspected.

Investigators are pursuing several strategies to identify susceptibility genes for schizophrenia. These strategies include linkage studies, sib-pair analyses, and association studies. The possibility of a potential linkage for schizophrenia on chromosome 22q was initially suggested by two groups independently working on genome searches (1–3), although neither group reported statistically significant linkage results. The fact that the same area of the genome (22q12–13) was suggestive of linkage in these two independent searches, as well as in a subsequent study using sib-pair analysis (4), justified further pursuit of the region. Indeed, further analyses by our group suggested that the most likely region of interest includes 22q11 (5) and that no region of chromosome 22q could be excluded. Furthermore, simulation studies suggested that if heterogeneity exists such that <25% of the families are linked to a locus in 22q11–13, then the currently available linkage data were not adequately powerful to draw firm conclusions about the area (6).

Given the suggestive results, region 22q11–13 was examined for diseases that map to this region and are known to share some phenotypic expression with schizophrenia (1). Velocardiofacial syndrome (VCFS), a congenital malformation syndrome, was identified. Clinical presentation of VCFS in children includes, with variable expression, cleft palate, heart anomalies, typical facies, and learning and attention problems (Online Mendelian Inheritance in Man, 1994). VCFS is part of a spectrum of phenotypes that include cardiac abnormalities, abnormal facies, T-cell deficit due to thymic hypoplasia, cleft palate, and hypocalcemia due to hypoparathyroidism. All are attributed to a microdeletion in the VCFS/DiGeorge sequence (DGS) critical region on chromosome 22q11.21–q11.23. It is not known whether the clinical phenotypes seen are due to hemizygosity of one gene or to deletion of a series of contiguous genes. When inherited, VCFS appears to be an autosomal dominant disorder. It has been hypothesized that DGS and VCFS may be etiologically the same (7).

With respect to psychiatric problems in VCFS, it has been observed that patients with the syndrome have "characteristic personality features" including blunt or inappropriate affect (8) and develop psychosis when they reach adolescence (9). The results of a recent study in which a group of randomly chosen adults diagnosed with VCFS were psychiatrically evaluated suggested that individuals with VCFS are at an increased risk to develop schizophrenia during late adolescence and early adulthood than individuals in the general population (10).

MATERIALS AND METHODS

Fluorescence *in Situ* Hybridization (FISH). Standard chromosome spreads were obtained from Epstein–Barr virus-transformed lymphoblastoid cell lines. Probes were labeled by nick-translation with biotin- or digoxigenin-dUTP (Boehringer Mannheim). After preannealing of the probe with human Cot-1 DNA, hybridizations were performed at 37°C in 2 × SSC/10% dextran sulfate/50% deionized formamide, and slides were washed to a stringency of 0.1× SSC at 60°C. Signals were developed with avidin–rhodamine or fluorescein isothiocyanate anti-digoxigenin antibody, and chromosomes were counterstained with 4′,6-diamidino-2-phenylindole. Images were captured and processed with a cytovision probe workstation (Applied Imaging, Pittsburgh). A minimum of 10 metaphases and 20 nuclei were examined for each analysis. Probe N25 was purchased from Oncor. Cosmids DO832 and c350 were kindly provided by Peter Scambler (Institute of Child Health, London) and control cosmid DAC9 was provided by Marc Lipinski (Centre National de la Recherche Scientifique, Paris).

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Abbreviations: VCFS, velocardiofacial syndrome; DGS, DiGeorge sequence; FISH, fluorescence *in situ* hybridization.
†To whom reprint requests should be addressed.

Somatic Cell Hybrids. Two fusion protocols were followed. The first involved a chromosome 22-specific selection method; permanent lymphoblastoid cell lines were established from each patient and 3×10^6 lymphoblasts were then fused with an equal number of Ade^-1 cells, a CHO (chinese hamster ovary) K1 purine auxotrophic mutant cell line, deficient in the enzyme adenylosuccinate lyase, which maps to human chromosome 22. (The Ade^-1 cell line was kindly provided by D. Patterson Eleanor Roosevelt Institute, Denver.) After fusion, the cells were exposed to adenine-free medium (after 24 hr), so that only the hamster cells that had incorporated human chromosome 22 survived. In the second protocol, CHTG 49 (Chinese hamster thioguanine 49) cells were used as the recipient cell line. Hypoxanthine/aminopterin/thymidine selection allowed survival of hybrids that had retained the X chromosome. We estimated that ≈40% of the hybrids had also cosegregated chromosome 22. The number of independent clones isolated varied from as few as 4 for some cell lines to 13 for others. After isolation, colonies were expanded to a sufficient number of cells and DNA was made. About 25% of the hybrids were found to have retained both chromosomes 22 and were not included in the analysis. One hybrid was found to be broken, having retained only the distal part of the deleted chromosome 22, and two were found to be contaminated; these three hybrids were also discarded from the analysis.

PCR Analysis. PCR was performed with the thermostable enzyme *Taq* polymerase (1.5 units per sample) (AmpliTaq; Perkin–Elmer/Cetus) and a programmable PCR apparatus (MJ Research, Watertown, MA). Target sequences were amplified in a 25-μl reaction mixture containing 100 ng of genomic hybrid DNA in 50 mM KCl/10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$/5 pmol of each primer/100 mM each dNTP (dATP, dCTP, dGTP, dTTP)/0.01% gelatin. Amplification was for 35 cycles; each cycle consisted of a 30-sec denaturation at 94°C, 1 min of annealing at 52°C, and a 1-min extension at 72°C. The final extension step was prolonged for 7 min. For all $(CA)_n$ dinucleotide probes, one of the primers was 5′-end-labeled with $[\gamma\text{-}^{32}P]ATP$, and the PCR products were resolved by electrophoresis on 6% denaturing polyacrylamide gels and detected by overnight autoradiography with an intensifying screen. For typings of sequence tagged sites (STSs), PCR was performed as described above, but none of the primers was labeled; 10 μl of the amplified product was subjected to electrophoresis in either 3% NuSieve gels or 8% nondenaturing polyacrylamide gels, depending on the size of the product. Gels were then stained with ethidium bromide and photographed. Most primer pairs for the polymorphic markers, as well as for the STSs (11) used in this study, were purchased from Research Genetics (Huntsville, AL). Markers D22S942, D22S947, D22S933, D22S935, D22S936, and D22S938 and their relative order are described by Morrow *et al.* (12).

RESULTS

In the present investigation, the possibility of genotypic overlap between schizophrenia and VCFS is examined in a series of two studies. In study A, we tested the hypothesis that we could identify 22q11 deletions among schizophrenic patients. In study B, we tested the hypothesis that a more extensive deletion of 22q11 may be present in VCFS patients who are also diagnosed with schizophrenia when compared to VCFS patients who have not been diagnosed as schizophrenic.

Study A. *Population.* Patients diagnosed with schizophrenia were selected from the Maryland epidemiologic sample, a systematic sample of patients with psychotic disorders who were hospitalized in the greater Baltimore area between 1983 and 1989 (13). A total of 695 patients in the Maryland sample were assigned a research diagnosis (DSM-III criteria; ref. 14) of schizophrenia, schizoaffective disorder, or schizophreniform disorder. For study A, we evaluated a randomly chosen subset of these patients who, in addition to being diagnosed with schizophrenic disorders, met the following criteria: (*i*) allowed us to recontact them; (*ii*) lived in Baltimore City and the surrounding counties; and (*iii*) were willing to participate in this study. Patients were asked to donate a small blood sample and allow a member of the research team to take several photographs of their face, ears, and hands. The photographs were taken to rate the presence of VCFS-like characteristics (typical facies; low-set ears; long, slender digits). The majority of the patients were male (78%). Most of the patients (92%) were assigned a DSM-III diagnosis of schizophrenia; five patients have a diagnosis of schizoaffective disorder and three patients have a diagnosis of schizophreniform disorder. The average age at onset of psychotic symptoms was 20.8 years (range, 5–41 years); 14% of the patients have a positive family history of psychotic illness. An additional schizophrenic patient with VCFS features (referred to here as GVA1418) was referred to the Cantonal Hospital in Geneva to one of us (S.E.A.) and was also included in study A.

Detection and initial characterization of 22q11 deletions in schizophrenic patients. This population was screened for 22q11 interstitial deletions. Two deletions among the Maryland epidemiology sample were identified and initially character-

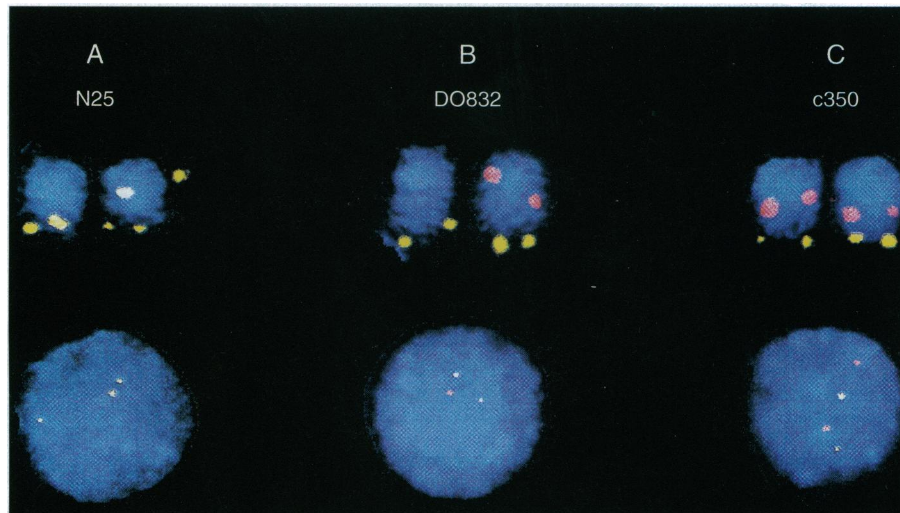

FIG. 1. FISH analysis of cell line AP1037. Probes for the deleted region were N25 (*A*), DO832 (*B*), and c350 (*C*). The 22qter control cosmid in all cases was DAC9. (*Upper*) Chromosomes 22. (*Lower*) Interphase nuclei.

ized. Both subjects were hemizygously deleted for cosmids N25 (D22S75) and DO832 (D22S502) (Fig. 1 *A* and *B*), as well as for cosmid sc11.1 (15), as detected by FISH. GVA1418 was also found to carry a hemizygous deletion for cosmid sc11.1. All these cosmids map within the extended VCFS/DGS critical region (16–18). Cosmid sc11.1, in particular, recognizes two distinct loci, A and B, that map proximal and distal to N25 and DO832, respectively (16–18) (see Table 3). Both loci were hemizygously deleted. The deleted subjects were all female and all had a history of learning problems as children. Examination of photographs of these individuals suggested some dysmorphic facial features consistent with a VCFS phenotype. Neither schizophrenic subject had a positive family history of schizophrenia or other psychotic disorders (Table 1).

To characterize the identified 22q11 interstitial deletions and determine their size, we developed somatic cell hybrid cell lines from lymphocytes from two of the schizophrenic subjects carrying the hemizygous 22q11 deletions (AP1046 and AP1037). Use of somatic cell hybrids allows us to segregate the deleted chromosome 22 from its normal counterpart, thus enabling us to study each chromosome separately, eliminating the background provided by the nondeleted chromosome. By using a battery of polymorphic markers from outside the deletion, we characterized the chromosomal contents of each hybrid clone and selected clones carrying intact single autosomes 22. The selected hybrid clones along with the genomic DNA from each patient were typed by PCR with 37 markers that cover the entire chromosome: 21 microsatellite markers of the dinucleotide repeat, $(CA)_n$ type, and 16 STS markers, as illustrated in Fig. 2.

This analysis showed that one deleted schizophrenic subject (AP1046) has a deletion defined proximally by D22S427 and distally by D22S636 (WI-362) (Fig. 2; Table 2) and including the deletion of D22S264. For the second deleted schizophrenic subject (AP1037), however, we find both copies of D22S264 to be present, suggesting that the distal breakpoint lies proximal to this marker (Fig. 2; Table 2). The presence of a smaller deletion in one of the schizophrenic subjects (AP1037) was confirmed by FISH, as indicated by the presence of cosmid c350 (D22S695) (18) in both copies (Fig. 1*C*; Table 3). GVA1418 was found, through FISH examination, to harbor a deletion that extends to include cosmid c350 (D22S695) (Table 3). (This deletion is, however, smaller than the deletion harbored by AP1046, based on information derived from

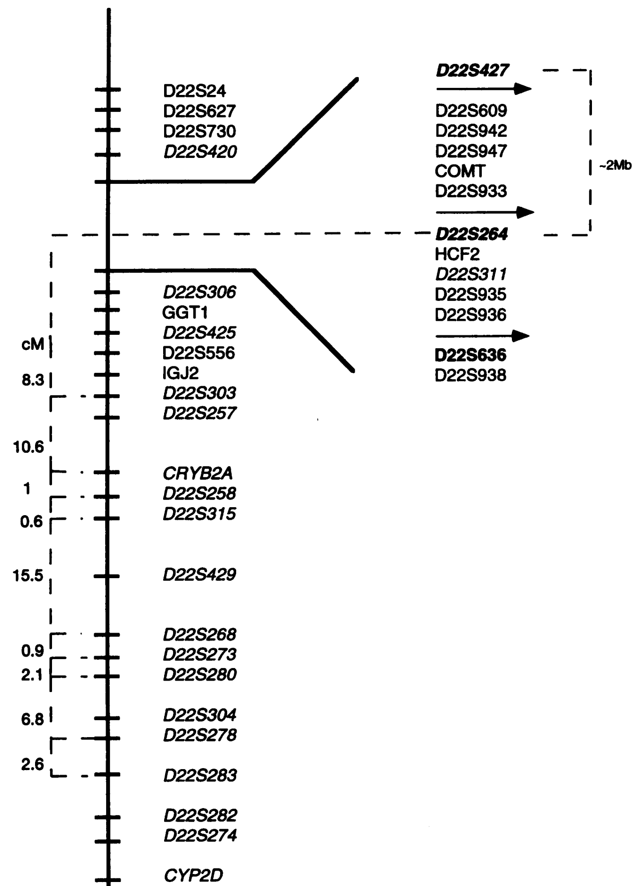

FIG. 2. Ideogram of chromosome 22q depicting all the markers tested on hybrid clones to characterize interstitial 22q11 deletions. All markers with bars on the chromosome were present in all cases in both copies. Italics denote microsatellite markers of the $(CA)_n$ type. Markers in boldface represent the most proximal (D22S427) and the most distal (D22S264 and D22S636) of the nondeleted markers in the patients tested. Arrows indicate deletion breakpoints. The region between D22S427 and D22S264 is the smallest region identified as critical for the schizophrenic phenotype. Map is not to scale. Top to bottom, centromere to telomere. cM, centimorgan(s).

Table 1. Patient characteristics

Patient	Sex	Age at VCFS diagnosis, years	Age at 1st psychotic episode, years	Current age, years	Clinical features
				Study A	
AP1046	F	N/A	32	34	Schizophrenia, classic facial VCFS features
AP1037	F	N/A	20	27	Schizophrenia, classic facial VCFS features, alcohol abuse, substance dependence
GVA1418	F	N/A	31	38	Schizophrenia, classic facial VCFS features
				Study B	
AP1012	M	30	21	31	Classic facial VCFS features, submucous cleft palate, schizophrenia, simple phobia
AP1014	F	14	26	31	Classic facial VCFS features, cleft palate, schizophrenia, obsessive compulsive disorder
AP1011	F	17	N/A	31	Classic facial VCFS features, submucous cleft palate, small ears, learning disability, alcohol dependence
AP1017	F	4	N/A	23	Classic facial VCFS features, submucous cleft palate, Raynaud phenomenon
AP1013	M	27	N/A	29	Classic facial VCFS features, cleft palate, small ears, alcohol abuse
AP1016	M	3	N/A	17	Classic facial VCFS features, submucous cleft palate, hypocalcemia

N/A, not applicable.

Table 2. Map of 22q11 deletions characterized in this study

Marker	Patient							
	AP1046	***AP1037***	**AP1012**	**AP1014**	AP1011	AP1017	AP1013	AP1016
D22S427	+	+	+	+	+	+	+	+
D22S609	−	−	−	−	−	−	+	+
D22S942	−	−	−	−	−	−	+	+
D22S947	−	−	−	−	−	−	+	+
COMT	−	−	−	−	−	−	+	+
D22S933	−	−	−	−	−	−	+	+
D22S264	−	+	−	−	−	−	+	+
HCF2	−	+	−	−	−	−	+	+
D22S311	−	+	−	−	−	−	+	+
D22S935	−	+	−	−	−	−	+	+
D22S936	−	+	−	−	−	−	+	+
D22S636	+	+	+	+	+	+	+	+
D22S938	+	+	+	+	+	+	+	+

The four patients in boldface characters have been diagnosed as schizophrenics. The two in boldface italics are from the Maryland epidemiologic sample (study A). +, Presence of marker in both copies; −, absence of one copy.

microsatellite analysis; M.K., unpublished data.) Thus, the "critical" region for the schizophrenic phenotype on 22q11, based on these three patients, is now defined by microsatellite marker D22S264 and cosmid c350 (D22S695) distally and by microsatellite marker D22S427 proximally, a region roughly estimated to be 1.5–2 Mb.

Study B. *Population.* Postadolescent VCFS patients were drawn from a group of patients known to the Montefiore Center for Craniofacial Disorders (New York) and involved in a pilot study in which the psychiatric status of VCFS patients and their relatives was systematically examined (10). Briefly, the patients were chosen according to the following criteria: (*i*) a diagnosis of VCFS based on clinical information; (*ii*) currently over the age of 15 years; (*iii*) living in the metropolitan New York area and available (i.e., not hospitalized); and (*iv*) willing to participate in a psychiatric examination. The sample included 18 VCFS patients, 4 of whom were found to meet the DSM-III-R criteria for schizophrenia. The prevalence of schizophrenia in this small sample (22%) is much higher than the prevalence of schizophrenia in the general population (1%) (10).

Characterization of the VCFS deletions associated or not with schizophrenia. This sample allowed us to test the hypothesis that a more extensive deletion may be present in the subgroup of VCFS patients (who carry a 22q11 interstitial deletion) and also have a diagnosis of schizophrenia. We randomly chose two from the subgroup of four VCFS patients also diagnosed as schizophrenics and four from the subgroup of 14 VCFS patients that upon psychiatric examination were found to be free of psychosis and from these six patients (Table 1) generated somatic cell hybrid cell lines. We chose a panel of 30 independent hybrid clones to study. These hybrid clones along with the genomic DNA from all six patients were typed with 37 markers that cover the entire chromosome: 21 microsatellite markers of the dinucleotide repeat $(CA)_n$ type and 16 STS markers, as illustrated in Fig. 2. Fig. 2 and Table 2 summarize the results from analysis of the deletions. Twenty-seven of the markers were present in both copies in all six patients and showed no evidence of hemizygosity (Fig. 2, horizontal bars). Ten were found to be hemizygously deleted in four patients and no marker was found to be homozygously deleted in any patient (Table 2). Two VCFS patients were found not to harbor any detectable deletions on 22q11 with any of the markers tested, a finding consistent with the observation that only ≈76% of VCFS patients are hemizygous for part of 22q11 (19).

Interestingly, in all four deleted patients, we find the deletions to be defined identically by D22S427 proximally and D22S636 (WI-362) distally. We mapped the proximal breakpoint between marker D22S427 (present in both copies in all patients) and marker D22S609 (WI-326) (hemizygously deleted in all patients carrying deletions). The distance between these two markers is estimated to be ≈300 kb, based on the presence of both markers on the same yeast artificial chromosome (YAC) (12). The distal breakpoint was mapped between marker D22S936 (hemizygously deleted in all patients carrying deletions) and D22S636 (WI-362) (present in both copies in all patients). Based on the average resolution of 60 kb in the region containing these two markers, it can be estimated that the distance between them is ≈120 kb (12).

Taken together, our results suggest that distally no correlation exists between the extent of the deletion and the additional schizophrenic phenotype, since there is at least one schizophrenic patient (study A) with a more proximally located breakpoint (breakpoint proximal to D22S264 and cosmid c350) than two patients who have 22q11 deletions and phenotypically are free of any psychosis. Proximally, under the current scrutiny and the relative sparsity of markers from the region between D22S427 and D22S609 (WI-326), it remains unclear whether there are differences in the extent of the deletions as they correlate to the phenotype. However, even if such differences existed proximally, they would be confined between markers D22S427 and D22S609, a distance of <300 kb, based on their presence within the same YAC.

The size of the four VCFS deletions included in study B is estimated to be >2 Mb (see above). This deletion size is observed in ≈85% of VCFS/DGS patients (17). However, a small proportion of VCFS/DGS patients carry smaller deletions and have helped to define the shortest region of overlap to ≈300 kb, a region that lies in the proximal end of the larger deletions and does not extend beyond the catechol-*O*-

Table 3. FISH analysis of schizophrenic subjects

Patient	(Proximal)	sc11.1A	N25 (D22S75)	DO832 (D22S502)	sc11.1B	c350 (D22S695)	(Distal)
AP1046		Deleted	Deleted	Deleted	Deleted	Deleted	
AP1037		Deleted	Deleted	Deleted	Deleted	Present	
GVA1418		Deleted	ND	Deleted	Deleted	Deleted	

ND, not done.

methyltransferase gene (17) (Fig. 2). This 300-kb region is included within the commonly deleted region in all schizophrenic patients in this study.

DISCUSSION

We have previously reported that there is a higher incidence of schizophrenia among VCFS cases (10). A prediction from this observation is that 22q11 deletions would be identified in populations of schizophrenic patients. This prediction is realized in the studies reported here. Although the exact frequency of the 22q11 deletion is not known in the general population, an estimate of the prevalence (based on the frequency of congenital heart disease caused by 22q11 deletions) is 1/4000 (0.025%) (20). In our sample, 2 of 100 (or 2%) of the schizophrenic patients examined so far by FISH using the probe sc11.1 (15) carry the deletion. This represents a minimum estimate of 22q11 mutations associated with schizophrenia. The exact risk for schizophrenia associated with 22q11 deletions will be determined only after more extended studies involving larger numbers of patients and control subjects are conducted. However, the observation presented here, together with the higher incidence of schizophrenia among VCFS cases, previously reported (10), are consistent with the hypothesis of increased susceptibility to schizophrenia associated with 22q11 deletions.

Haploinsufficiency for a gene or genes at 22q11 has been associated with a variety of clinical diagnoses, including DGS, VCFS, and less severe but related dysmorphologies including familial congenital heart disease. There seems to be a common underlying embryological basis—namely, a deficiency in the contribution of the migrating neural crest population to development of the structures derived from the 3rd branchial arch and 4th pharyngeal pouch system (7). Hemizygosity of 22q11 is now being associated with at least some type of schizophrenia. Interestingly, schizophrenia is believed by many researchers to be a neurodevelopmental disorder (21, 22) and a number of postmortem studies describe consistent findings of neuronal migratory abnormalities (23–27).

Two major questions need to be addressed: (*i*) Why do interstitial deletions of 22q11 increase the risk for schizophrenia? (*ii*) Why are <100% of the 22q11 microdeletions associated with psychosis? Our results render the possibility of more extended deletions associated with schizophrenia unlikely, especially distally. Other hypotheses that could explain why not all subjects with interstitial 22q11 deletions develop schizophrenia include genomic imprinting, additional mutations (germ line or somatic) in the existing single copy of the gene(s) responsible for the schizophrenic phenotype, existence of a modifying locus or loci located elsewhere in the genome, or environmental influences. It is important to screen additional populations of schizophrenic patients for 22q11 deletions and identify and psychiatrically evaluate a larger number of VCFS patients 20 years old and over in an effort to define the shortest region of overlap. This effort will help us narrow the list of genes that could be considered candidates for schizophrenia and therefore warrant further analysis. Although the exact frequency of schizophrenia cases associated with 22q11 lesions remains to be determined, identification and characterization of the relevant genes from this region will hopefully provide valuable insights into the pathogenesis of the disease.

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