Recurrent CNVs Disrupt Three Candidate Genes in Schizophrenia Patients

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Schizophrenia is a severe psychiatric disease with complex etiology, affecting approximately 1% of the general population. Most genetics studies so far have focused on disease association with common genetic variation, such as single-nucleotide polymorphisms (SNPs), but it has recently become apparent that large-scale genomic copy-number variants (CNVs) are involved in disease development as well. To assess the role of rare CNVs in schizophrenia, we screened 54 patients with deficit schizophrenia using Affymetrix's GeneChip 250K SNP arrays. We identified 90 CNVs in total, 77 of which have been reported previously in unaffected control cohorts. Among the genes disrupted by the remaining rare CNVs are *MYT1L*, *CTNND2*, *NRXN1*, and *ASTN2*, genes that play an important role in neuronal functioning but—except for *NRXN1*—have not been associated with schizophrenia before. We studied the occurrence of CNVs at these four loci in an additional cohort of 752 patients and 706 normal controls from The Netherlands. We identified eight additional CNVs, of which the four that affect coding sequences were found only in the patient cohort. Our study supports a role for rare CNVs in schizophrenia susceptibility and identifies at least three candidate genes for this complex disorder.

Schizophrenia [MIM 181500] is a severe psychiatric disease with complex etiology. It is characterized by a variety of psychotic symptoms, including delusions and hallucinations, reduced interest and drive, altered emotional reactivity, and disorganized behavior.[1] Despite the facts that schizophrenia occurs at a high frequency (~1%) in the general population and that heritability is around 0.80, genetic studies so far have largely failed to identify susceptibility factors that confer risk alleles for the disease.[2,3] This indicates that schizophrenia is a clinically and genetically heterogeneous disease. Among the strongest candidate genes are *DISC1* [MIM 605210] and genes in the 22q11 region, such as *COMT* [MIM 116790] and *PRODH* [MIM 606810], which have been initially identified as rare, high-penetrant cytogenetic variants that either disrupt or completely delete the genes involved.[4,5] Genomic microarrays allow for the systematic genome-wide analysis of a more subtle form of cytogenetic variation, i.e., copy-number variation (CNV): genomic deletions and/or duplications of 1 kb to 3 Mb in size.[6] Recent microarray studies have identified many such CNVs in a variety of complex disorders, including mental retardation,[7,8] autism spectrum disorder,[9–12] and schizophrenia.[13–18] It has now become evident that rare CNVs can have important implications in the etiology of schizophrenia. Although rare CNVs may be related to psychiatric diseases, little is known about the identity of the genes affected by these variants, and even less is known about the frequency at which these individual CNVs occur in the different disorders.[19] Here, we combined a genome-wide CNV screen in patients with deficit schizophrenia, with a targeted, but considerably larger, follow-up study in a general-schizophrenia patient–control cohort. The deficit subtype of schizophrenia is characterized by primary, enduring, negative symptoms and is chronic in course.[20] Therefore, patients with such a severe subtype of schizophrenia provide a good reference set for the detection of CNVs, of which recurrence assessment and further characterization can be done in the larger cohort.

We initially performed a genome-wide screen for CNVs on a small discovery cohort of 54 Dutch patients diagnosed with deficit schizophrenia, according to the Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR,[21] for whom extensive phenotypic information was available from medical records and interviews, including the Comprehensive Assessment of History and Symptoms (CASH)[22] and the Schedule for Deficit Syndrome (SDS).[20] We hybridized genomic DNA of these patients to Affymetrix's GeneChip 250K SNP (Nsp) arrays, according to standard protocols (GEO accession number: GSE12714), and identified genome-wide CNV using the Copy Number Analyzer for Affymetrix GeneChip (CNAG) v2.0 software.[23] We set the Hidden Markov Model (HMM) algorithm to calculate copy numbers on the basis of four consecutive SNPs, with an estimated false-positive rate of 5%.[24] We identified a total of 90 CNVs, with an average genomic size of 353 kb (varying from 9 kb to 1.7 Mb; Table S1, available online). Most of these CNVs

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DOI 10.1016/j.ajhg.2008.09.011.

(85%, n = 77) were previously reported by Redon et al.[6]—who used the same Affymetrix platform to study CNVs in 270 unaffected HapMap individuals—or observed by us and listed in our internal CNV database. The latter contains data from genomic microarray analyses on more than 400 unaffected individuals of European descent. The occurrence of these CNVs in these relatively small control cohorts strongly indicates that they occur frequently in the general population and do not represent rare genomic variants underlying disease.[6] Previously, we and others[7,14–17] have shown that rare CNVs might be responsible for a significant proportion of common neurological disorders. For this reason, we focused on the rare CNVs as an underlying cause of schizophrenia.

We identified in our discovery cohort 13 rare CNVs (Table S2) not described by Redon et al.[6] and neither present in our internal, unaffected control database nor previously observed in our diagnostic genome-wide microarray studies encompassing more than 1300 patients with unexplained mental retardation. The HMM algorithm detected twelve variants, but visual inspection and subsequent validation revealed one additional CNV that contained too few SNPs for the algorithm to detect it (12 SNPs in a 98 kb region on chromosome 9; Figure 1). Some of the rare variants do overlap with regions listed in the Database of Genomic Variants (DGV); however, most variants detected in our patients extended beyond those reported in the DGV. Each of the CNVs was detected in one patient only, illustrating the rare nature of these variants. Seven rare variants did not affect any gene, nor were they located in highly conserved regions. No information on whether the CNVs are inherited or have arisen de novo in these patients is available. On the basis of the gene content, we selected and validated four CNVs by either multiplex ligation-dependent probe amplification (MLPA) or genomic quantitative PCR, essentially as described previously[13,25] (Figure 1B, Table S5, Table S6). The four CNVs included two duplication CNVs in chromosomal regions 2p25.3 and 5p15.2 and two deletions in chromosomal regions 2p16.3 and 9q33.1 (Figure 1). The deletions (237 and 98 kb in size) each affect a single gene: *NRXN1* [MIM 600565] on 2p16.3 and *ASTN2* on 9q33.1. The duplications were considerably larger (967 and 930 kb) and affect four genes on 2p25.3 and seven genes on 5p15.2, respectively. Interestingly, the breakpoints of both duplications disrupt the terminal parts of two genes: *MYT1L* at 2p25.3 and *CTNND2* [MIM 604275] at 5p15.2. These observations suggest that, besides dosage variation, these CNVs might also have disruptive effects, as has been reported before.[17,26] The two genes affected by the deletions, *NRXN1* and *ASTN2*, as well as the genes at the breakpoints of the two duplications, *MYT1L* and *CTNND2*, are highly expressed in the brain as compared to several other tissues[27] (Table S3), and they have all been implicated in neuronal functioning, adhesion, and migration.[28–31]

Table 1 displays detailed demographic and diagnostic information of the four index patients. All patients were diagnosed with schizophrenia of the paranoid type, and all fulfilled the deficit syndrome criteria, according to the SDS. All four patients were hospitalized and have been on antipsychotic drugs for several years. At the time of inclusion, all four patients were severely ill, with considerable delusions and hallucinations. Additionally, they displayed aggressive behavior during psychosis and fulfilled criteria for formal thought disorder and physical anergia.

In a second-phase analysis, we studied CNV within these four regions in a larger cohort, consisting of 752 additional patients with schizophrenia and 706 unaffected control individuals. The patients were recruited from a variety of psychiatric hospitals and institutions in The Netherlands, partly coordinated via academic hospitals in Amsterdam, Groningen, Maastricht, and Utrecht (The Genetic Risk and Outcome of Psychosis [GROUP] project). All patients had been diagnosed for subtypes of schizophrenia according to the DSM-IV-TR.[21] The controls were volunteers and were all screened for any psychiatric history, the majority via the CASH. Both cases and controls were of Dutch descent and they all gave informed consent, as did the patients of the first phase group. The study was approved by the Ethics Committee of UMC Utrecht and by the appropriate local institutional review boards at all other participating hospitals. Genomic DNA of all patients and controls was hybridized to HumanHap550v3 BeadArray (Illumina, San Diego, CA, USA) according to standard protocols. CNVs were included only if they were detected by two different software packages, PennCNV[32] and QuantiSNP.[33] We analyzed these data for CNVs in the four candidate regions, with boundaries extended to include the entire coding and noncoding sequence of genes that were disrupted in the index patients. Within the candidate regions, we detected CNVs in seven additional patients and in one control (Figure 1, Table S4, Figure S1). Four of the CNVs identified in the patient cohort were located within the *NRXN1* gene at 2p16.3, one of which affected exonic sequences. In addition, two previously unreported CNVs were identified at 2p25.3: a 3.8 Mb duplication affecting 11 genes, including *MYT1L*, and a 56 kb duplication not affecting any known gene. Finally, a 360 kb duplication that affected part of both the *ASTN2* and *PAPPA* [MIM 176385] genes was observed in the 9q33.1 region. The single CNV identified in the control cohort is an intronic duplication within the *NRXN1* gene (Figure 1). Table S4 displays phenotypic characteristics of these patients; the control subject with the duplication within the *NRXN1* gene was free of any clinical psychological features.

Clearly, most CNVs in our study were observed in *NRXN1*; three deletion CNVs affecting coding sequences and three that were entirely intronic (two duplications and one deletion). Even though Redon et al.[6] observed one unaffected HapMap individual with a deletion in *NRXN1*, deletions in this gene have generally been linked to neurological disorders, such as mental retardation,[34] autism,[11,12,35,36] and schizophrenia.[14,17] All of the

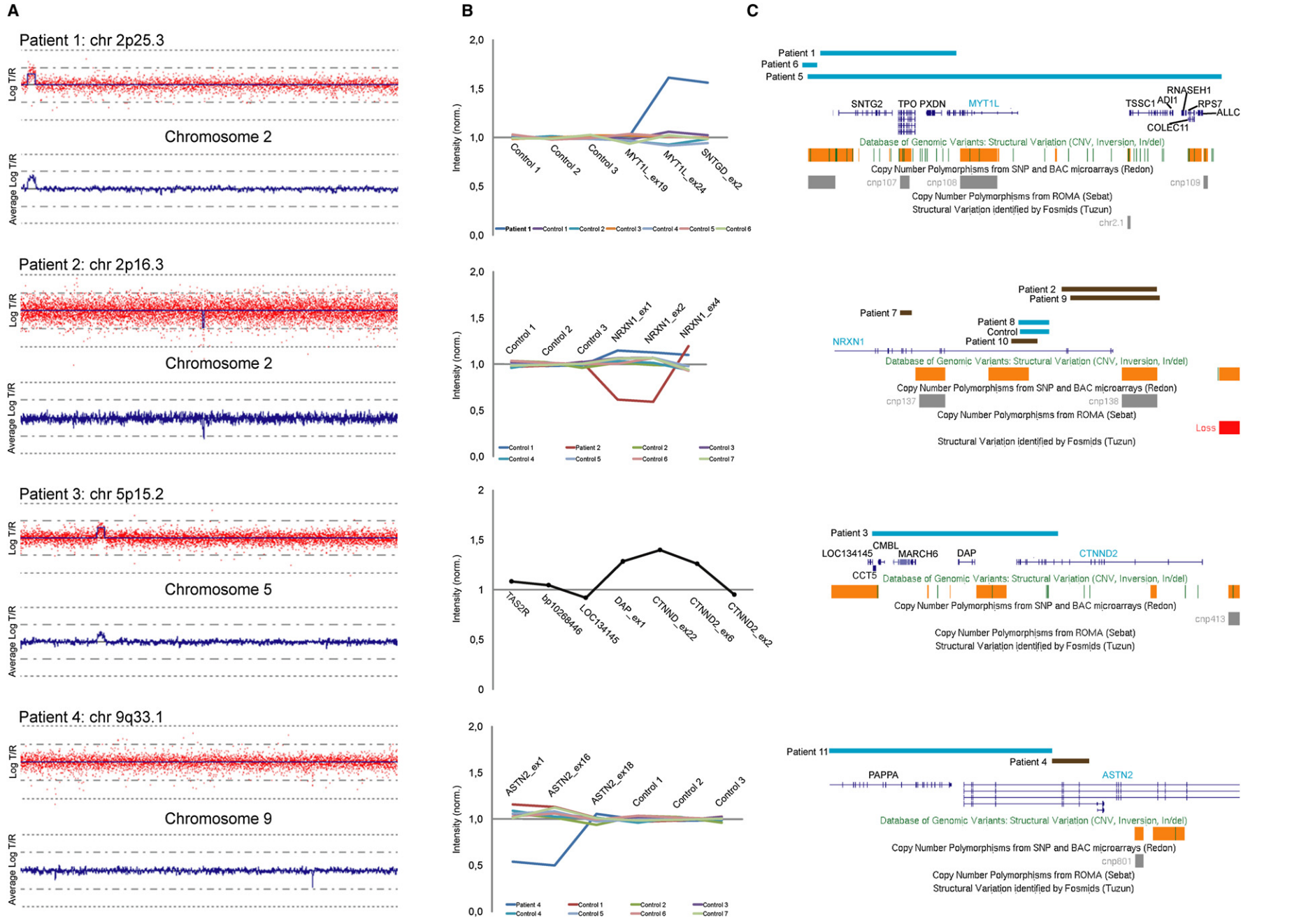
A
Patient 1: chr 2p25.3
Log T/R
Chromosome 2
Average Log T/R
Patient 2: chr 2p16.3
Log T/R
Chromosome 2
Average Log T/R
Patient 3: chr 5p15.2
Log T/R
Chromosome 5
Average Log T/R
Patient 4: chr 9q33.1
Log T/R
Chromosome 9
Average Log T/R
B
Intensity (norm.)
Control 1
Control 2
Control 3
MYT1L_ex19
MYT1L_ex24
SNTGD_ex2
Patient 1
Control 4
Control 5
Control 6
NRXN1_ex1
NRXN1_ex2
NRXN1_ex4
Patient 2
Control 7
TAS2R
bp10268446
LOC134145
DAP_ex1
CTNND_ex22
CTNND2_ex6
CTNND2_ex2
Patient 3
ASTN2_ex1
ASTN2_ex16
ASTN2_ex18
Patient 4
C
Patient 1
Patient 6
Patient 5
SNTG2
TPO
PXDN
MYT1L
RNASEH1
TSSC1
AQI1
RPS7
ALLC
COLEC11
Database of Genomic Variants: Structural Variation (CNV, Inversion, In/del)
Copy Number Polymorphisms from SNP and BAC microarrays (Redon)
cnp107
cnp108
cnp109
Copy Number Polymorphisms from ROMA (Sebat)
Structural Variation identified by Fosmids (Tuzun)
chr2.1
Patient 2
Patient 9
Patient 7
Patient 8
Control
Patient 10
NRXN1
cnp137
cnp138
Loss
Patient 3
LOC134145
CMBL
MARCH6
DAP
CTNND2
CCT5
cnp413
Patient 11
Patient 4
PAPPA
ASTN2
cnp801

Table 1. Detailed Clinical Features of Four Index Patients with CNVs

		Patient 1	Patient 2	Patient 3	Patient 4
Demographics	Sex	Male	Male	Female	Male
	MPA	Facial and toe syndactyly	-	-	-
	MR	Mild MR	Yes	-	Yes
	Other	-	-	Stereotypical movements	Epilepsy
Diagnostics	DSM-IV Diagnosis (according to CASH)	Paranoid type (295.30)	Paranoid type (295.30)	Paranoid type (295.30)	Paranoid type (295.30)
	Age of onset	18	20	20	26
	Core symptoms	Severe delusions/ hallucinations of sexual/ aggressive content	Isolation, chaotic and aggressive behavior	Severe delusions/ hallucinations; very aggressive behavior	Bizarre delusions, anhedonia, paranoid/ somatic delusions; aggressive behavior
	Fulfilling SDS criteria (total score if present)*	Yes	Yes	Yes	Yes (12)
	Additional information	Physical anergia; formal thought disorder	Physical anergia; formal thought disorder	Physical anergia; suicidal; formal thought disorder	Physical anergia; formal thought disorder
CNV	Chromosome	2p25.3	2p16.3	5p15.2	9q33.1
	Size (kb)	1014	389	1019	98
	Type	Duplication	Deletion	Duplication	Deletion
	No. of genes	4	1	7	2
	Candidate gene	*MYT1L*	*NRXN1*	*CTNND2*	*ASTN2*
	Gene function	Neural cell differentiation, nervous-system development	Synaptic formation and maintenance, axon guidance	Cell motility, dendritic branching, neuron adhesion	Glial-guided neuron migration, axonal guidance

* For six items, there can be a score ranging from 0–4.

apparently pathogenic deletions reported so far affect the first few exons of the *NRXN1* gene, including the promoter region. In addition, rare missense mutations in exon 1 have been associated with autism.[37] In our study, we encountered two patients with deletions affecting the promoter region and exon 1, as well as a deletion affecting exons 8–10. The CNVs in the other two patients and in one unaffected control were located in intronic sequences of which any relations to disease cannot as yet be discerned (Figure 1 and Table S4). Similarly, the larger duplications in the 2p25.3 region, affecting many genes, are more likely to be causative than is the 56 kb intergenic duplication observed in this region. The *MYT1L* gene located in this region is a member of the myelin transcription factor 1 family, which regulates proliferation and differentiation of oligodendrocytes[38] by regulating transcription activity in the CNS.[31] Although *MYT1L* itself has not previously been linked to disease, a deletion in its homolog, *MYT1*[MIM 600379], was recently observed in a patient with mental retardation.[39] In addition, Law et al.[40] hypothesized that *MYT1* regulates *NRG1* [MIM 142445] expression in schizophrenia patients. We hypothesize that the (partial) duplications of *MYT1L* in patients 1 and 5 in our study cohort might affect this regulatory function in the CNS by either disruption or dosage effects. For *ASTN2* at 9q33.1, an exonic deletion was observed in patient 4 and a partial duplication was observed in patient 11, suggesting that disruption of this gene might have functional consequences. The other gene affected by the duplication in patient 11—pregnancy-associated plasma protein A (*PAPPA*)—is predominantly expressed in the placenta[41] and, thus, not likely to be involved in the development of schizophrenia. As yet, little is known about *ASTN2*—except that it is highly expressed in the brain (Table S3)—but its homolog, *ASTN* [MIM 600904], is known to play an important role in neuronal migration.[29,42] Besides the CNV in 5p15.2 that disrupted the *CTNND2* gene in patient 3, no additional aberration was identified within this locus, suggesting that CNVs in this locus are extremely rare and, thus, likely to be pathogenic. Interestingly, *CTNND2* is located in the critical region for autism spectrum disorder[43] [MIM 209850]) and mental retardation[44] in Cri du Chat syndrome[45] [MIM 123450].

In conclusion, we identified rare CNVs affecting four neuronal genes in a selected cohort of 54 schizophrenia patients. A subsequent targeted but considerably larger

Figure 1. Four Rare CNVs in Patients with Schizophrenia

(A) CNAG plots of deletions and duplications in four patients. Upper x axis: Log2 test-over-reference ratios of hybridization signals of individual SNPs (red dots) and the corresponding HMM algorithm (blue line). Lower x axis: moving average (ten SNPs) result of the individual Log2-ratios.

(B) MLPA (patients 1, 2, and 4) and qPCR (patient 3) validation of all four CNVs.

(C) Location of duplications and deletions in the 11 patients and the control subject in the respective genomic regions. Displayed are duplications (blue horizontal lines) and deletions (brown horizontal lines), affected genes (blue), and adjacent known variants from the Database of Genomic Variants and from studies of unaffected subjects.

follow-up study did not reveal any coding-sequence-affecting CNVs in these loci in the control cohort, whereas four were found in the patient cohort. In total, we identified four small exonic deletions disrupting a part of a gene implicated in neuronal functioning, as well as three large duplications with possibly similar disruptive effects. The frequency of these rare CNVs combined is considerable in the selected cohort of Dutch patients with deficit schizophrenia (4 of 54 patients, 7% of cases). Even in the larger, unselected Dutch cohort of schizophrenia patients, we identified these rare CNVs in seven out of 752 cases (0.9%), even though we screened only 5.7 Mb of genomic sequence. The frequency of rare CNVs affecting genes reported by Walsh et al.[17] in patients with schizophrenia or schizoaffective disorder is much higher (24% of cases), but the authors also identified such variants at considerable frequency in controls (5%). Although these results from us and those from others[15–17] demonstrate the relevance of rare CNVs in schizophrenia, they also challenge the clinical interpretation of nonrecurrent rare CNVs, even if the CNVs affect genes. Our findings indicate that it is important to carefully interpret the individual CNVs in order to assess their involvement in disease. Large-scale CNV-based association studies are essential for unraveling the role of CNVs in disease etiology, but they should take into consideration that the consequences of intronic or intragenic CNVs are likely to be different from those of CNVs that affect coding sequences. Small, single-gene CNVs are different from larger CNVs affecting many genes, and deletions can clearly exert different effects than duplications. Finally, assessing inheritance patterns of CNVs and their segregation with disease will be of added value for establishing the true clinical relevance of this important class of genomic variation.

Supplemental Data

Supplemental Data include one figure and six tables and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

We are grateful to the patients for their cooperation. We thank Joseph DeYoung for technical assistance, Harry van Someren for database management, and Christian Gilissen for bioinformatic support. This work was supported by a grant from The Netherlands Organization for Health Research and Development (ZON-MW, to J.A.V.). The GROUP project was also supported by a grant from ZON-MW, within the Mental Health program (project number: 10.000.1001). In addition, genome-wide SNP genotyping of the Dutch schizophrenia sample was funded by the National Institute of Mental Health grant RO1 MG078075 to R.A.O.

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Received: July 2, 2008
Revised: September 10, 2008
Accepted: September 15, 2008
Published online: October 9, 2008

Web Resources

The URLs for data listed herein are as follows:

Database of Genomic Variants: http://projects.tcag.ca/variation/
Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/
Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/Omim/
ZonMw: http://www.zonmw.nl/en/programmes/all-programmes/mental-health/

Accession Numbers

The GEO accession number for the CNV microarray data reported in this paper is GSE12714.

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