Failure to Confirm CNVs as of Aetiological Significance in Twin Pairs Discordant for Schizophrenia

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Copy number variations (CNVs) are common structural variations in the human genome that strongly affect genomic diversity and can play a role in the development of several diseases, including neurodevelopmental disorders. Recent reports indicate that monozygotic twins can show differential CNV profiles. We searched CNVs in monozygotic twins discordant for schizophrenia to identify susceptible loci for schizophrenia. Three pairs of monozygotic twins discordant for schizophrenia were subjected to analysis. Genomic DNA samples were extracted from peripheral blood lymphocytes. We adopted the Affymetrix Genome-Wide Human SNP (Single Nucleotide Polymorphism) Array 6.0 to detect copy number discordance using Partek Genomics Suite 6.5 beta. In three twin pairs, however, validations by quantitative PCR and DNA sequencing revealed that none of the regions had any discordance between the three twin pairs. Our results support the hypothesis that epigenetic changes or fluctuation in developmental process triggered by environmental factors mainly contribute to the pathogenesis of schizophrenia. Schizophrenia caused by strong genetics factors including copy number alteration or gene mutation may be a small subset of the clinical population.

Keywords: CNVs, schizophrenia, genotype, monozygotic twin, epigenetic change

Schizophrenia is a chronic, debilitating psychiatric illness with a 1% worldwide prevalence. Genetic studies have shown that schizophrenia has a high heritability, with strong genetic factors involved in its etiology. Twin studies have played an important role in the elucidation of the genetic factors underlying neurodevelopmental disorders. Several twin studies have revealed that the concordance rate between monozygotic twins is 41–79% for schizophrenia, whereas the concordance rate between dizygotic twins is 0–14% (Shih et al., 2004; Kakiuchi et al., 2008). The higher concordance rate in monozygotic rather than dizygotic twins for schizophrenia suggests the contribution of genetic factors. Phenotypically discordant monozygotic twins are especially interesting resources for genetic studies, and twin studies could facilitate the identification of the causative genes of phenotypes. Kondo et al. (2002) reported that a nonsense mutation in *IRF6*, which is a causative gene for Van der Woude syndrome, was found in one affected individual in monozygotic phenotypically discordant twins. In relation to neurodevelopmental disorders, Bruder et al. (2008) reported that discordant monozygotic twins with parkinsonism showed different copy number variation (CNV) profiles.

CNVs are the most prevalent type of structural variations in the human genome that largely contribute to genomic diversity. Redon et al. (2006) and Carter et al. (2007) showed that as much as 12% of the human genome and thousands of genes are variable in copy number. A great number of CNVs may not be pathogenic but simply contribute to human genome diversity not related to phenotype. Meanwhile, some CNVs have been proven a significant factor related to disease susceptibility. Recent studies reported that CNVs contribute to genetic vulnerability factors and can play an important role in the etiology of several neurodevelopmental disorders (Sebat et al., 2007; 2009). Xu et al. (2008) found that de novo copy number mutations were about eight times more frequent in patients with sporadic schizophrenia. Numerous copy number analyses in schizophrenia revealed that genes that were disrupted by CNVs, which include *TBX1*, *ERBB4*, *SLC1A3*, *RAPGEF4*, *CIT*, *NRXN1*, and 16p11.2 region, were candidate genes and regions for schizophrenia (Cook et al., 2008; McCarthy et al., 2009; Merikangas et al., 2009; Walsh et al., 2008); however, most of these are rare copy number variants and the contribution of those genes to schizophrenia is restricted to a tiny part of etiologies.

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To date, numerous causative genes for schizophrenia have been identified; however, because of genetic heterogeneity, there is still a long path to the elucidation of the pathogenesis of schizophrenia. To identify causative genes for schizophrenia, we have utilized the Affymetrix Genome-Wide Human SNP Array 6.0 in three pairs of monozygotic twins discordant for schizophrenia. Here, we describe the results of CNV and genotype profiles in three pairs of monozygotic twins.

Methods

Subjects

Three pairs of monozygotic twins discordant for schizophrenia participated in this study. Ten years had passed after the onset of schizophrenia in the affected individuals in all twin pairs. All of the twins were male, and their mean age was 53 years old. Two well-trained psychiatrists diagnosed the twins by structured clinical interview, and all affected individuals corresponded to the DSM-IV-TR criteria for the undifferentiated type of schizophrenia.

DNA Microarrays

Ten ml of peripheral blood samples was collected after obtaining written informed consent, and genomic DNA was extracted from blood lymphocytes using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

DNA microarray experiments were performed using Affymetrix Genome-Wide Human SNP Array 6.0 (SNP Array 6.0) (Affymetrix, Santa Clara, CA, USA). We performed a paired analysis for loss of heterozygosity (LOH) and an unpaired analysis for copy number analysis using control individuals' data. All of the computer analyses were performed using Genotyping Console (Affymetrix) and Genomics Suite version 6.5 beta software (Partek, St. Louis, MO, USA). Genomic copy number data were analyzed with Partek Genomics Suite software using a segmentation algorithm with stringent p value cutoff.

Quantification of Genome Copy Number

We performed real-time quantitative PCR using an intercalating dye, SYTO13 (Molecular Probes, Eugene, OR, USA), which is an alternative to SYBR green I, or using Universal Probe Library (Roche Diagnostics, Mannheim, Germany) to verify copy number changes suggested by the microarray analyses. Primers and probes were designed using the website software Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com). Real-time PCR amplification was run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). All samples were measured in tetraplicates.

DNA Sequencing

To verify the SNP genotypes obtained by SNP Array 6.0, we performed direct sequencing of PCR-amplified genomic DNA fragments including SNPs that showed discordant allele calls in each twin pair. The amplified fragments were directly sequenced after purification with exonuclease I and NTPhosTM Thermolabile Phosphatase (Epicentre, Madison, WI, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit and run on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using Variant Reporter (Applied Biosystems) and ATGC version 6.0 (Software Development, Tokyo, Japan).

Results

Microarray Analysis Results

Quality control (QC) data obtained from the SNP Array 6.0 are summarized in Table 1. The call rate and contrast QC in SNP Array 6.0 data were > 95% and > 1.50, respectively, for all samples, and both values indicated experiments using the SNP Array 6.0 were done well.

Copy number analysis of microarray data using Partek Genomics Suite showed some deleted or amplified regions in each twin pair (data not shown). Regions with discordant genotyping between twins from microarray data are summarized in Table 2.

Unpaired analysis of 6 individuals in comparison with ethnically-matched normal controls (HapMap-JPT) revealed that an approximately 3 kb region within the *SLC25A37* gene was deleted in two of the three schizophrenia twin pairs, 11A/B and 31A/B. The deleted region (chromosome 8:23460969–23463786) was not registered in the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Quantitative PCR Results

We verified the copy number state by real-time PCR of the regions with discordant copy number, including genes, by paired analysis using SNP Array 6.0. Primers were designed for the middle position of the regions.

Table 1

Summary of Twin Samples and Affymetrix GeneChip Genotyping Results

Samples	Sex	Phenotype	SNP call rate	Contrast QC*
11A / B	Male	Schizophrenia/unaffected	99.444 / 99.516	2.38 / 2.48
21A / B	Male	Schizophrenia/unaffected	98.974 / 99.175	1.88 / 2.22
31A / B	Male	Schizophrenia/unaffected	99.199 / 99.179	2.26 / 1.60

Note: *Contrast QC (Quality Control) is per sample Quality Control test metric for SNP Array 6.0 intensity data. In high-quality data sets, the Contrast QC metric is higher than the 0.4 threshold according to user manual provided by the manufacturer.

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Table 2

List of Loss of Heterozygosity Regions Derived from Microarray Data

chr. #	Physical position		Twin #	Validated SNPs	Overlapping genes
	Start	End			
1	4309356	4465925	11A / B	rs7521665, rs4654438	LOC284661
	45006976	45050681	31A / B	rs6676749	BEST4, PLK3, RPS8, SNORD38A, SNORD38B, SNORD46, SNORD55
	170792582	170870563	31A / B	rs2472550	Region overlaps with 70.55% of C1orf9
2	50182138	50311147	31A / B	rs1452762, rs6712119	Contained within NRXN1
	142093343	142097262	31A / B	rs355581	Contained within LRP1B
3	3693732	3821526	31A / B	rs7613060, rs769806	Region overlaps with 4.23% of LRRN1
	123371895	123393318	31A / B	rs1501900	Region overlaps with 37.81% of CASR
4	24093201	24162064	31A / B	rs4697063	Region overlaps with 34.68% of DHX15
	81368193	81418990	11A / B	rs10518238, rs1458046	Region overlaps with 24.07% of FGF5
	101451872	101646851	31A / B	rs3756037	Region overlaps with 57.10% of EMCN
	109080142	109167540	31A / B	rs4395588	Region overlaps with 15.93% of CYP2U1 and 42.51% of HADH
	126258785	126764905	31A / B	rs7660602	FAT4
5	38382422	38389445	11A / B	rs9292705	Contained within EGFLAM
	166816487	166823787	31A / B	rs17068499	Contained within ODZ2
6	35297977	35376388	31A / B	rs3800385	ZNF76, region overlaps with 3.59% of DEF6 and 36.49% of SCUBE3
	119363250	119468737	31A / B	rs6913082	Contained within FAM184A and 74.19% of FAM184A
8	86383578	87077669	31A / B	rs1845891, rs1553015, rs6605618	CA1, CA2, CA3, REXO1L1, REXO1L2P
9	207826	208183	31A / B	rs10964703	Contained within DOCK8
	3900136	3920251	31A / B	rs630219	Contained within GLIS3
	7154039	7156090	31A / B	rs1556100	Contained within KDM4C
	112777053	112781741	31A / B	rs3758281, rs16915618	Contained within LPAR1
10	68497020	68657339	31A / B	rs10822972	Contained within CTNNA3, region overlaps with 21.12% of LRRTM3
	100181485	100219522	31A / B	rs11599112	Region overlaps with 28.02% of HPSE2 and 39.99% of HPS1
11	8896463	9040536	11A / B	rs4929922	C11orf17, region overlaps with 29.17% of SCUBE2
	19449860	19466526	31A / B	rs11820210	Contained within NAV2
12	21894811	21895465	31A / B	rs4148673	Contained within ABCC9
	33716220	36801139	31A / B	rs11052835, rs2387324	ALG10
	38818800	39404433	21A / B	rs7132869	LRRK2, region overlaps with 5.43% of CNTN1
	63692809	63739310	11A / B	rs4964104	Region overlaps with 18.58% of WIF1
	69385261	69392041	31A / B	rs10879183	Contained within PTPRR
	77123022	77139445	31A / B	rs9971904	Region overlaps with 48.10% of NAV3
	120088239	120155175	31A / B	rs25643	Region overlaps with 29.88% of P2RX7 and 34.55% of P2RX4
13	102227169	102252370	31A / B	rs9514058	KDELC1, region overlaps with 11.79% of BIVM
16	13150832	13161027	31A / B	rs4781419	Contained within SHISA9
22	24570234	24607029	31A / B	rs6004793	Contained within MYO18B
	36847351	36893417	31A / B	rs2076116	Contained within PLA2G6

Note: Chr. # means the number of chromosome.

Quantitative PCR was performed for a total of 120 regions. However, we could not reconfirm the differences between twins in all 120 tested regions. In addition, quantitative PCR within the *SLC25A37* gene revealed no loss or gain of the genome in comparison with ethnically matched control individuals.

Sequencing Results

DNA sequencing was performed for a total of 37 regions surrounding SNPs that had shown discordant genotype calls from microarray analysis within twin pairs. We selected one or more SNP(s) called discordant genotype in each LOH region. Sequencing revealed all of the SNPs were concordant between twin pairs (data not shown).

Discussion

In this study, we analyzed genomic alterations, CNVs and genotypes, in three pairs of monozygotic twins discordant for schizophrenia. None of the regions of copy number difference between twins shown by SNP Array 6.0 were reverified by quantitative PCR, and none of the genotype discordance was reverified by sequencing. Additionally, no novel CNVs was detected in the identified CNVs between twins. To our knowledge, this is the first report verifying the data from high-density and high-resolution DNA microarrays by quantitative PCR and DNA sequencing. Our results indicate that genomic alterations including CNVs and gene mutations contribute minimally to etiologies of

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schizophrenia. The large genome-wide study by The Wellcome Trust Case Control Consortium (WTCCC) revealed CNVs is not main cause of bipolar disorder, which is one of the neurodevelopmental disorders (WTCCC, 2010). This report have a different concept from our study because our study aimed to find copy number alteration as a single gene disorder, however, WTCCC report could not discover the CNV contributing to the bipolar disorder. Our results may support the hypothesis that epigenetic changes (Roth et al., 2009), which can influence the expression of genes without affecting the DNA sequence, mainly contribute to the pathogenesis of schizophrenia.

SNP Array 6.0 allows us to detect different genotype or copy number neutral LOH regions. In our twin comparison, copy number neutral LOH would indicate segmental uniparental disomy (UPD) in twin pairs. Actually, UPD of the paternal allele at 11p15 in the affected twin caused discordance for hemihypertrophy in monozygotic twins (West et al., 2003). Furthermore, recent studies revealed that UPD was associated with schizophrenia. UPD on chromosome 1 and 5q32-qter in a patient with schizophrenia has been described in 2004 and 2006, respectively (Abecasis et al., 2004; Seal et al., 2006). But no genotype difference between twins was confirmed in this study.

Here, we presented no genomic discordance between twins; hereinafter, we will discuss some speculation about the relation between genetic factors and phenotypic discordance. First, it is possible that mosaicism at specific tissues (i.e., brain) because of postzygotic genomic rearrangements causes discordant phenotypes between monozygotic twins. Although we used DNA samples extracted from peripheral blood cells in this study, mosaic genomic rearrangement could be detected in brain. It is clear that the ideal source for studies of neurodevelopmental disorders is brain tissue. Nonetheless, it is practically impossible to harvest the brain tissue of twins (Kato et al., 2005). Olfactory sensory neurons have recently been shown to be easily accessible neuronal cells that have been useful for studies on schizophrenia (Arnold et al., 2001), enabling the study of neuronal cell character including genotype and copy number state. Second, it is possible that smaller-scale genomic aberrations below detection sensitivity influence the discordant phenotype of monozygotic twins. SNP Array 6.0 is one of the highest resolution platforms commercially available and allows us to identify CNVs much smaller than 10 kb. However, McCarroll et al. (2008) showed that the detection rate using the SNP Array 6.0 sharply diminished for CNVs <4 kb. To increase sensitivity, the use of many more detection probes is needed, and more than one experimental platform should be performed in future studies.

Bruder et al. (2009) successfully detected many copy number changes in peripheral blood using a Bacterial Artificial Chromosome (BAC)-array at mosaic state (~20%) in nine monozygotic twins discordant for parkinsonism. All of the nine pairs had copy number discordancy in their reports. Because their results suggested copy number change could be found in the mosaic state, tissue-specific mosaicism is a possible explanation for psychiatric disorders. We may have overlooked copy number change in a mosaic state in peripheral blood with the use of the SNP Array 6.0 instead of the BAC-array because the SNP Array 6.0 is a powerful tool to identify small regions with copy number change but is not suitable to detect copy number in a mosaic state.

It seems most likely that epigenetic changes between monozygotic twins influence the phenotypic discordance of monozygotic twins. Several recent studies indicate that epigenetic changes contribute to the etiology of schizophrenia. Rett syndrome and Fragile X syndrome are neurodevelopmental disorders caused by a single gene defect and dysregulation of DNA methylation very early in life (Amir et al., 1999; Das et al., 1997). Kaminsky et al. (2009) have shown that differences in DNA methylation profiles increase in monozygotic twins along with aging. Because the onset of schizophrenia is later than Rett syndrome and Fragile X syndrome, it is possible that cumulative epigenetic modifications could be one cause of schizophrenia development. Furthermore, a recent study by Roth et al. (2009) suggested that DNA methylation and histone modification triggered by influence of environmental factors is responsible for the difference in onset age between these disorders. Akbarian et al. (2005) indicated that histone modification contributes to the pathogenesis of prefrontal dysfunction in patients with schizophrenia based on the finding that the level of H3-(methyl)arginine 17 in patients with schizophrenia exceeded control values by 30%. Thus, genome-wide DNA methylation and genome-wide histone modification studies for monozygotic twins discordant for phenotypes may be promising techniques in future twin studies. In fact, Baranzini et al. (2010) reported genomic sequence and epigenetics (methyl-cytosine) analysis of monozygotic twin discordant for multiple sclerosis using next generation sequencer. They could not find reproducible differences between twins, but these comprehensive analyses including genome and epigenome sequence are just started. As Crow (2002) discussed, it is important to analyze the genetic and epigenetic influence to the species-specific characteristics. Comprehensive genetic and epigenetic analysis of discordant monozygotic twins will be advanced using next generation technologies.

In summary, we did not detect genomic alterations including CNVs and gene mutations between twins discordant for phenotype. Our results indicate that schizophrenia caused by genomic alterations may be a small subset of the clinical population and may support the hypothesis that epigenetic mechanisms triggered by the influence of environmental factors are associated with the etiology of schizophrenia. Experimental investigations of epigenetic mechanisms

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such as expression analysis, methylation site sequence and histone modification studies using DNA samples extracted from olfactory sensory neurons are needed to identify the differences responsible for discordant phenotypes in future studies.

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