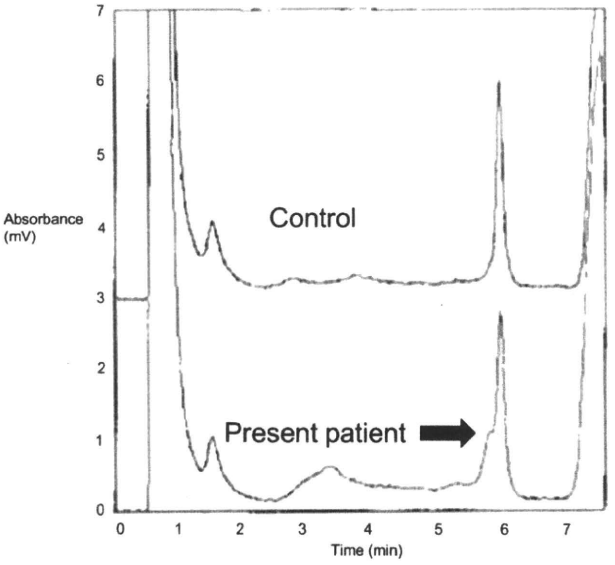

Fig. 3 Denaturing high-performance liquid chromatography of the 29th exon of the *NIPBL* gene (upper panel: control, lower panel: patient). Arrow shows the abnormal peak in the translation area (29th exon) of the *NIPBL* gene.

analyses in the various multiple malformation syndromes specifically associated with congenital diaphragmatic hernia are likely to shed light on which anomalies lead to diaphragmatic hernia.

In the present case, a mutation of C to T (nonsense mutation) at the 5524th base in the translation area of the *NIPBL* gene was identified. As a result, we concluded that this variation was likely to be the cause of the BDLS with diaphragmatic hernia. The *NIPBL* gene is located at 5p13.1 and contains 47 exons, and its transcription is thought to be related to Notch signal transmission. There have been many confirmed gene mutations, including deletion and insertion mutations, that are associated with BDLS (Gillis *et al.* 2004; Bhuiyan *et al.* 2006; Schoumans *et al.* 2007). Further, Musio *et al.* (2006) and Deardorff *et al.* (2007) have presented reports relating BDLS to both *SMC1* and *SMC3* gene mutations.

DNA analysis is important for confirming BDLS diagnosis. Analysis of gene mutations in genes such as *NIPBL* also represents a useful diagnostic method. With the accumulation of cases such as ours, further description of this disease will be possible.

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CLINICAL REPORT

AMERICAN JOURNAL OF
medical genetics

Co-Occurrence of Prader–Willi and Sotos Syndromes

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Received 13 January 2010; Accepted 5 May 2010

A patient with atypical phenotypes of Prader–Willi syndrome (PWS) was subjected to investigate genomic copy numbers by microarray-based comparative genomic hybridization analysis. Severe developmental delay, relative macrocephaly, protruding forehead, cardiac anomalies, and hydronephrosis were atypical for PWS. Concurrent deletions of 15q11-13 and 5q35 regions were revealed and identified as paternally derived. The sizes and locations of the two deletions were typical for both deletions. Although each deletion independently contributed to the clinical features, developmental disturbance was very severe, suggesting combined effects. This is the first report of co-occurrence of PWS and STS. The co-occurrence of two syndromes is likely incidental.

Key words: Prader–Willi syndrome; Sotos syndrome; aCGH

How to Cite this Article:
Okamoto N, Akimaru N, Matsuda K, Suzuki Y, Shimojima K, Yamamoto T. 2010. Co-occurrence of Prader–Willi and Sotos syndromes.
Am J Med Genet Part A 152A:2103–2109.

INTRODUCTION

Prader–Willi syndrome (PWS; OMIM #176270) is caused by deficiency of paternally expressed imprinted transcripts within chromosome 15q11-q13 [Ledbetter et al., 1981]. It is characterized by obesity, hypotonia, hypogonadism, and behavioral abnormalities [Holm et al., 1993]. Most paternal PWS deletions are bracketed by recurrent breakpoints (BP)1 or BP2 and BP3. Preturbed expression of genes including *SNURF–SNRPN* and multiple small nucleolar RNAs (*snoRNAs*) are associated with the clinical manifestations of PWS, but the specific contributions of individual genes are under investigation. Recent analysis revealed that deficiency of HBII-85 *snoRNAs* causes the key characteristics of the PWS phenotype, although some atypical features suggest that other genes in the region may make more subtle phenotypic contributions [Sahoo et al., 2008].

Sotos syndrome (STS; OMIM#117550) is an overgrowth syndrome characterized by pre- and postnatal overgrowth, macrocephaly, developmental delay, advanced bone age, and a distinctive face including frontal bossing, frontal sparseness of hair, hypertelorism, downslanting palpebral fissures, and pointed chin. Haploinsufficiency of the *NSD1* gene due to 5q35 microdeletions or intragenic mutations causes STS [Kurotaki et al., 2002]. Miyake et al. [2003] observed that microdeletions in STS are mostly of paternal origin. Common deletion breakpoints were located at two flanking low copy repeats (LCR), implying that non-allelic homologous recombination (NAHR) between LCRs is the major mechanism for the common deletion in STS [Kurotaki et al., 2005; Visser et al., 2005]. Central nervous system anomalies, cardiovascular and urogenital symptoms are more frequent in the microdeletion group [Nagai et al., 2003].

In this study, a patient with atypical phenotypes of PWS was subjected to investigate genomic copy numbers by microarray-based comparative genomic hybridization (aCGH) analysis. Concurrent deletions of 15q11-13 and 5q35 regions were detected and identified as paternally derived. Although each deletion independently contributed to the clinical features, growth and developmental disturbance were very severe, suggesting combined effects. This is the first report of co-occurrence of PWS and STS.

CLINICAL REPORT

A 14-year-old male propositus is the first-born child of healthy and non-consanguineous parents. After uncomplicated pregnancy, he was born at 39 weeks of gestation by induced delivery with overgrowth of length with 53 cm (90th centile).

Grant sponsor: Ministry of Health, Labour and Welfare in Japan.
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Published online 15 July 2010 in Wiley InterScience (www.interscience.wiley.com)
DOI 10.1002/ajmg.a.33544

His birth weight was within a normal limit as 3,010 g (25th centile). He was the first child of a 26-year-old mother and a 30-year -old father. Since cardiac murmur was found at birth, he was transferred to the neonatal intensive care unit and ventricular septal defect (VSD), atrial septal defect (ASD), and patent ductus arteriosus (PDA) were revealed by echocardiography. Micropenis and bilateral cryptorchidism were noticed. He had severe hypotonia and feeding difficulties in the early infantile period. Until his sucking improved at 6 months old, nasal tube feeding was required. Ultrasonography revealed bilateral vesicoureteral reflux and hydronephrosis. He showed a severe developmental delay with head control at 1 year of age and sitting alone at 6 years of age. He had generalized seizures at age 6 years. Electroencephalography revealed sporadic spikes at that time. Brain MRI showed no significant findings. He developed progressive obesity, as his weight was 10.0 kg (75th centile) at 9 months old of age and 12.4 kg (95th centile) at 1 year old of age. Conventional G-band chromosome analysis showed a normal male karyotype, and subsequent conventional FISH analysis for *SNRPN* revealed a deletion, indicating a diagnosis of PWS. In spite of that, relative macrocephaly, protruding forehead, frontal baldness, and mild overgrowth were atypical for phenotypic features of PWS (Fig. 1A). Although he was interested in food, hyperphagia was not prominent because of his restricted locomotive abilities. Gradually, his height SD scores decreased (Fig. 2). Partial growth hormone deficiency was found by endocrinological studies. When he was 14 years of age his bone age was measured at the 11-year-old level. His parents did not choose GH replacement therapy.

When we examined the patient at the age of 14 years, he showed severe mental retardation without vocalized words, muscular hypotonia, hypopigmentation, scoliosis, and distinctive facial features including protruding forehead; strabismus; hypertelorism; downslanting palpebral fissures; epicanthal folds; full cheeks; microstomia with downturned corners of the mouth; small hands with tapering fingers; and small feet (Fig. 1B). A wheel chair was required for him because his hip joint was unstable and he could not stand alone. His intelligent quotient (IQ) was measured by Kyoto Scale of Psychological Development as below 10. He was a calm and friendly boy. His interest in food became obvious, but self-injurious behaviors such as skin picking were not observed. Behavioral problems associated with STS including autistic spectrum disorder, hyperactivity, and aggression were not present. His weight was 29 kg (<3rd centile), and his length was 132 cm (<3rd centile) (Fig. 2). His head circumference was mean for his age. A comparison of typical features of PWS and STS and their clinical presentation in the patient are shown (Table I).

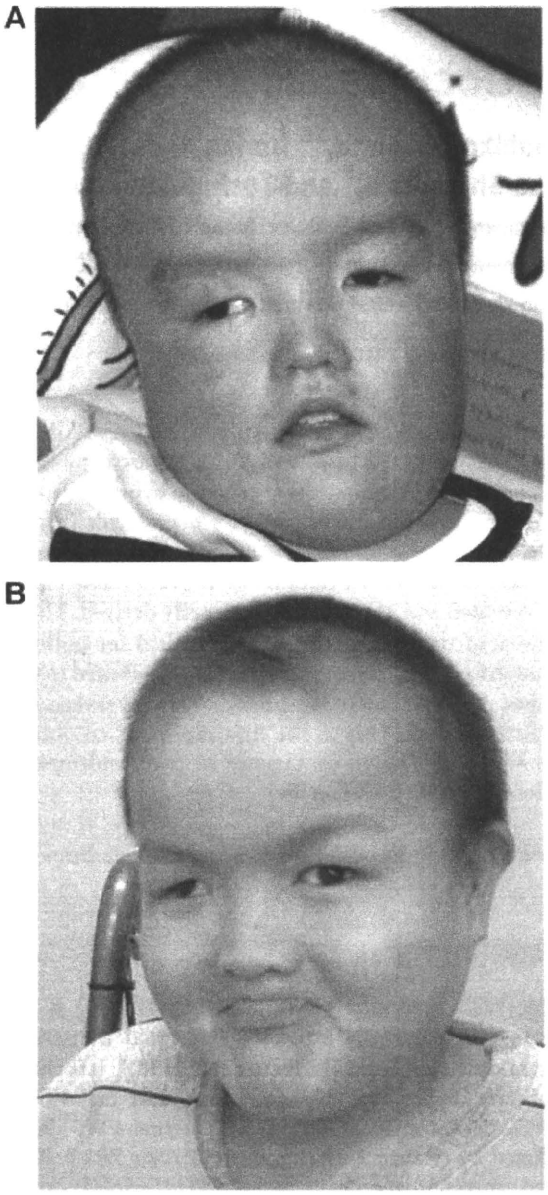

FIG. 1. Facial appearance of the patient at 6 years old (A) and 14 years old (B).

MATERIALS AND METHODS

After obtaining informed consents based on a permission approved by the institution's ethical committee, peripheral blood samples were obtained from the patient and his parents. Genomic DNAs were extracted using the QIAquick DNA extraction kit (QIAgen, Valencia, CA).

Based on the hypothesis that the patient might have an atypically larger deletion of chromosome 15 or have additional chromosomal aberrations, aCGH analysis was performed using the Human Genome CGH Microarray 60K (Agilent Technologies, Santa Clara, CA) as described previously [Shimojima et al., 2009].

Metaphase nuclei were prepared from peripheral blood lymphocytes by mean of standard methods and used for FISH analysis with human BAC clones selected from the UCSC genome browser (http://www.genome.ucsc.edu) as described elsewhere [Shimojima et al., 2009]. Physical positions refer to the March 2006 human reference sequence (NCBI Build 36.1).

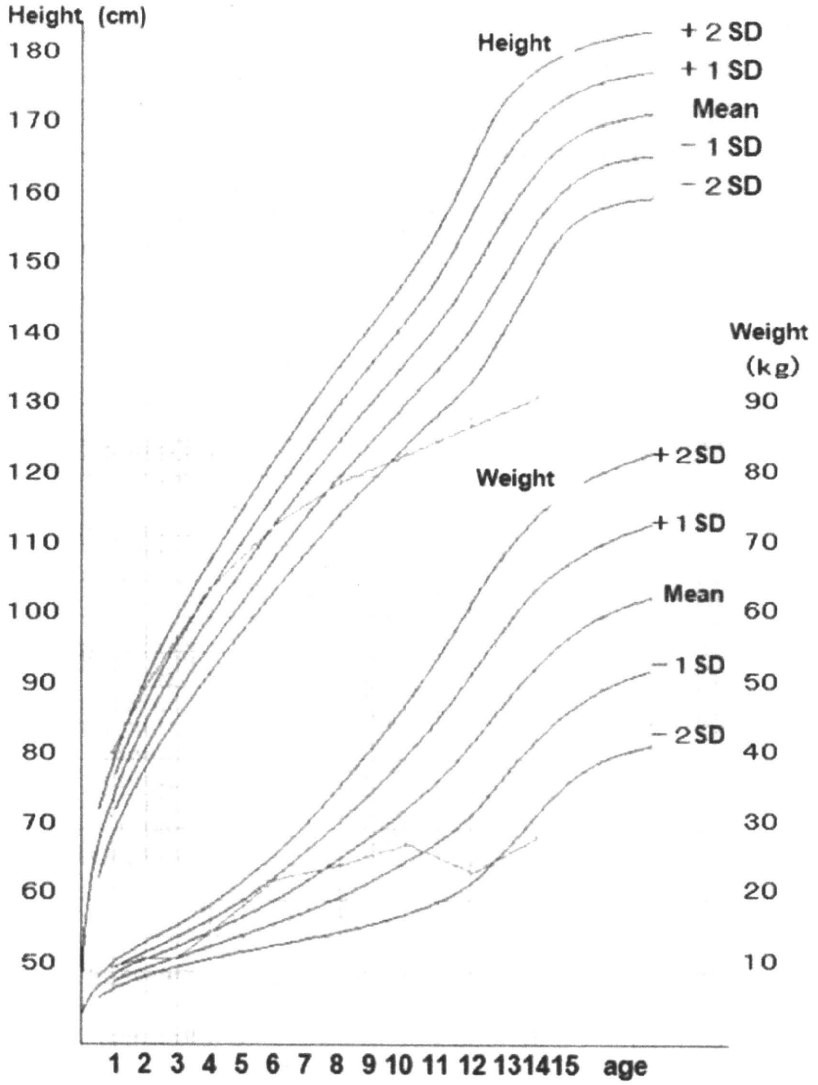

FIG. 2. Growth curve of the patient. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. A Comparison of Typical Features of PWS and STS and Their Clinical Presentation in the Current Patient

	Prader–Willi	Sotos	Current patient
Hypotonia	+	+	++
Mental delay	+	+	++
Hypopigmentation	+	−	+
Prominent forehead	−	+	+
Strabismus	+	+	++
Over growth	−	+	−
Growth delay	+	−	++
Obesity	+	−	+
Epilepsy	−	+	+
Congenital heart disease	−	+	+
Scoliosis	+	+	++
Hydronephrosis	−	+	+
Hypogonadism	+	−	+

+, common features; ++, prominent manifestations.

Microsatellite marker analysis was performed using the ABI Prism Linkage Mapping Set with D15S1002 and analyzed by GeneMapper (Applied Biosystems, Foster City, CA). In the deletion region of STS, no marker was available for the ABI Prism Linkage Mapping Set. Thus, the single-nucleotide polymorphisms (SNP) typing was carried out. From the STS deletion region of 5q35, eight SNPs, IMS-JST038690, IMS-JST087588, IMS-JST087589, IMS-JST183486, IMS-JST172005, IMS-JST073857, IMS-JST087921, and IMS-JST087922, were selected using in silico library, Japanese Single Nucleotide Polymorphisms (JSNP) database (http://snp.ims.u-tokyo.ac.jp/index.html). Allelic types were analyzed by PCR-direct sequencing method using the BigDye terminator (Applied Biosystems, Foster City, CA).

RESULTS

By aCGH analysis, loss of the genomic copy numbers was identified in the region of 15q11.2, which is responsible and typical for PWS (Fig. 3A). The concurrent deletion was

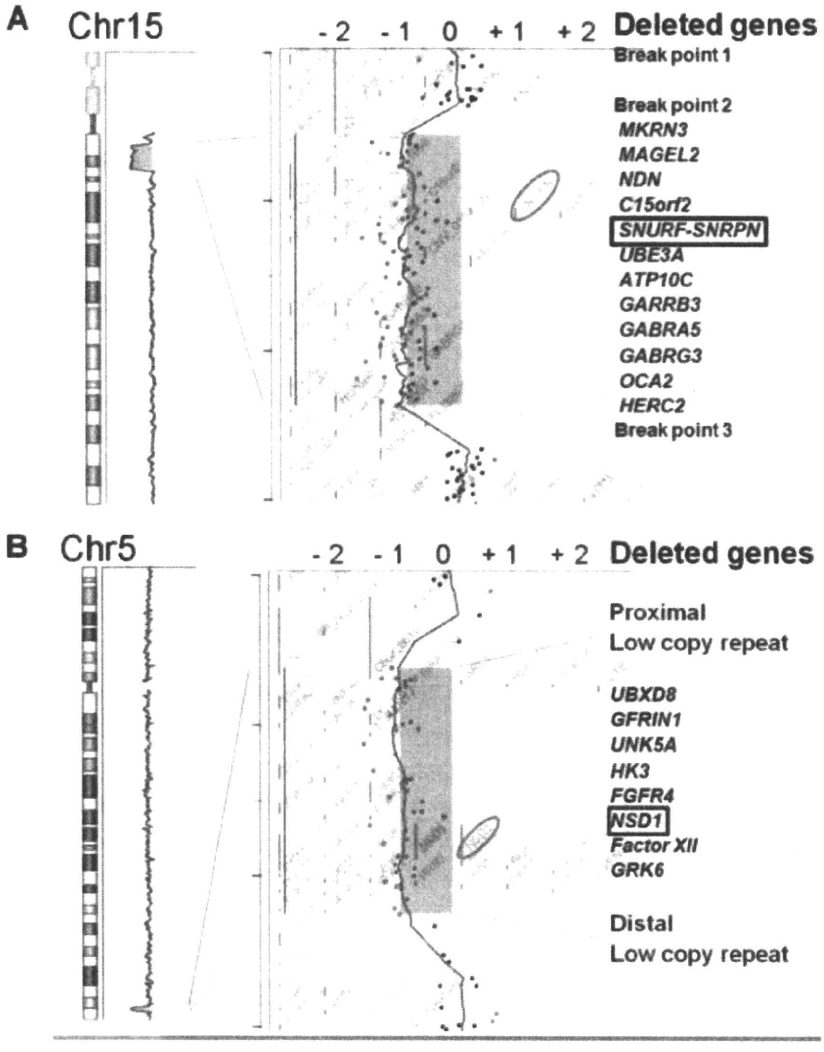

FIG. 3. aCGH profiles of the patient shown by CGH Analytics in Chromosome view (left) and Gene view (right). A: Typical deletion of PWS region including *SNRPN* is shown. B: Typical STS deletion including *NSD1* is indicated. The horizontal axis indicates the log 2 ratio of the genomic copy number. The blue rectangles indicate the regions containing copy number aberrations. The aberration areas are expanded in Gene view (right). The dots indicate the locations and the corresponding log 2 ratios of the probes. The red circles emphasize *SNRPN* and *NSD1*.

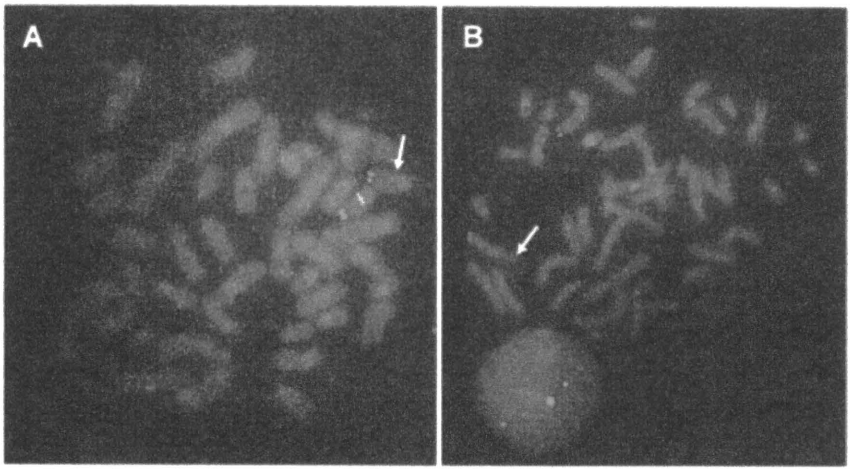

FIG. 4. FISH analysis to confirm the chromosomal deletion. A: One of the green signals covering *SNRPN*, RP11-1071C22 (15q11.2; 22601976–22822028), was deleted. Two red signals are the markers of chr15, RP11-48A4 (15q26.3; 99433829–99587322). B: One of the green signals covering *NSD1*, RP11-99N22 (5q35.2–5q35.3; 176474586–176655375), was deleted, whereas two red labeled RP11-94J21 (5p15.33; 1377471–1540913) signals were confirmed in all cells. Physical positions are referred to NCBI Build 36.1. White arrows indicate abnormal chromosomes in each FISH image.

identified in the region of 5q35, which is also responsible and typical for STS (Fig. 3B). FISH analyses confirmed the deletion of both regions (Fig. 4). There were no deletions of PWS region and STS region in both parents indicating de novo occurrence (data not shown).

To confirm the parental origin of both deletions, polymorphic markers were analyzed in the patient and his parents. Regarding the 15q11.2 region, the patient showed an only allele with 112-bp common to his mother, indicating the deletion of paternal allele (Fig. 5A). Among eight analyzed SNPs, only IMS-JST183486 was informative. The patient showed hemizyogous of T at the SNP position, whereas the father and the mother showed homozygous of A and T, respectively (Fig. 5B). From the result, we concluded that both deletions were derived from the paternal allele.

DISCUSSION

Initially, the patient was diagnosed as PWS due to severe hypotonia, hypopigmentation, hypoplastic genitalia, and small hands and feet. It was supported by hyperphagia and obesity which later developed. However, his facial features including relative macrocephaly, protruding forehead, frontal baldness, strabismus, downslanting palpebral fissures, and pointed chin were atypical for PWS. He also showed congenital cardiac anomalies, hydronephrosis, and epilepsy, which are rare findings in PWS. Severe hypotonia and severe developmental delay were also atypical for PWS. This was the reason why we analyzed genomic copy numbers.

To the best of our knowledge, this is the first report of co-occurrence of PWS and STS. Translocation between chromosomes 5 and 15 was excluded by G-banded analysis. Array CGH demonstrated that the sizes and locations of the two deletions were typical for both syndromes. Both of the deletions were derived from the paternal chromosome. We suspect that co-occurrence of two deletions is incidental.

His growth curve showed an interesting pattern. He showed overgrowth in the infantile period. Gradually, his growth velocity decreased. Now he shows severe growth deficiency. Although we understand that haploinsufficiency of *NSD1* might lead to height gain and patients with STS show advanced bone age, his growth deficiency was worse compared with standard PWS patients and his bone age was delayed [Nagai et al., 2000]. Growth hormone deficiency and severe scoliosis may explain his growth deficiency. We posit that each deletion contributed independently to the features. Severe growth and developmental delay might be explained by the combined effects of PWS and STS.

There are some reports of concurrent chromosomal aberrations in the same patients [Shimojima et al., 2009]. The result of this study indicates that there may be more frequent co-occurrences of two more deletions than what we think. When a patient shows atypical or overlapping features regardless of a previously established diagnosis, we would recommend investigation of whole genomic copy numbers by aCGH.

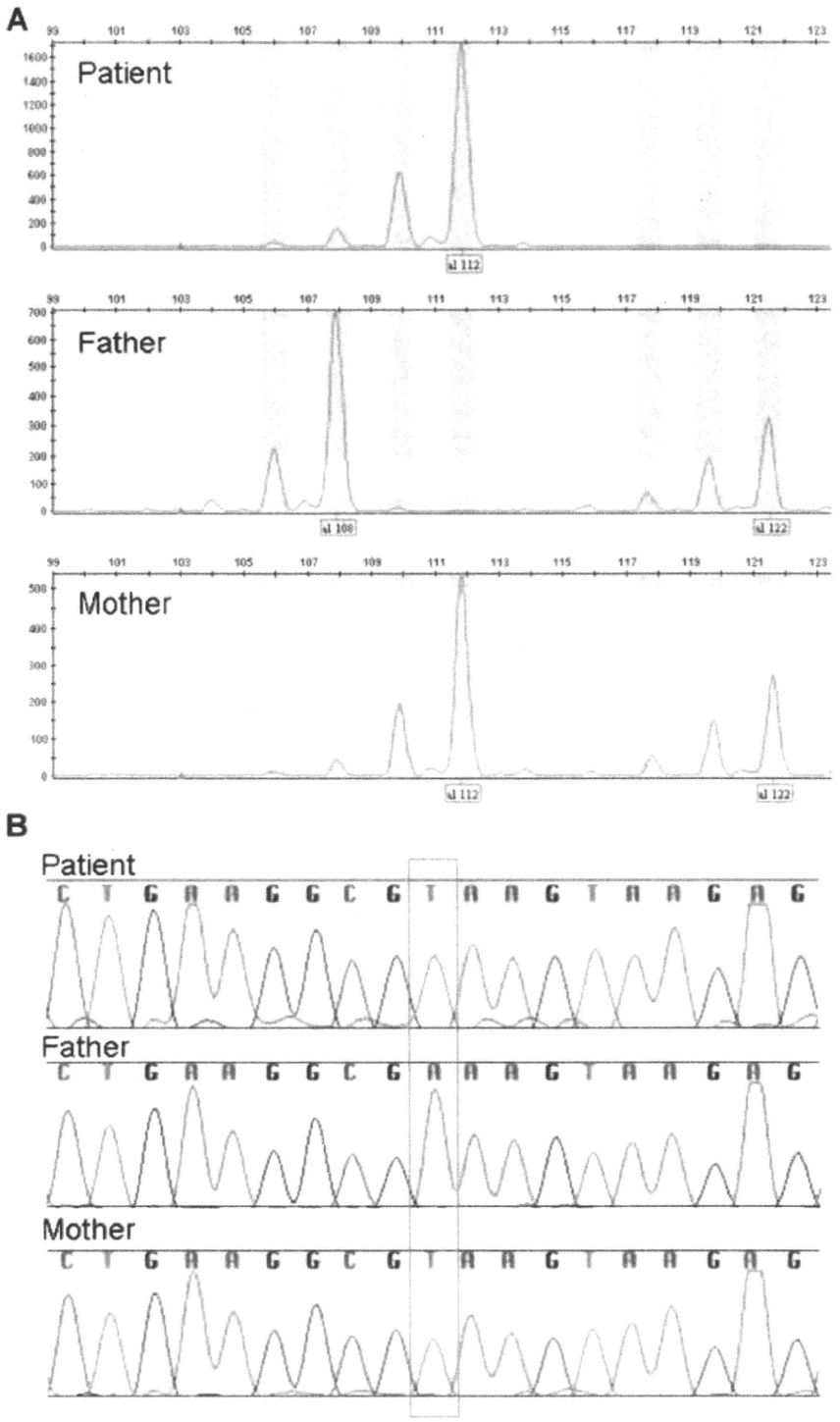

FIG. 5. Molecular analysis of the patient's family. A: GeneMapper analysis using D15S1002. The patient shows only one allele with 112-bp common with his mother, indicating the paternal deletion. B: SNPs analysis of IMS-JST183486. The patient's SNP type as T is only common with his mother, indicating the deletion of paternal allele.

ACKNOWLEDGMENTS

We thank the family for their co-operation. This study was supported by the Health and Labour Research Grants in 2009 by Ministry of Health, Labour and Welfare in Japan.

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Journal of Human Genetics (2011) 56, 110–124

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ORIGINAL ARTICLE

Clinical application of array-based comparative genomic hybridization by two-stage screening for 536 patients with mental retardation and multiple congenital anomalies

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Recent advances in the analysis of patients with congenital abnormalities using array-based comparative genome hybridization (aCGH) have uncovered two types of genomic copy-number variants (CNVs); pathogenic CNVs (pCNVs) relevant to congenital disorders and benign CNVs observed also in healthy populations, complicating the screening of disease-associated alterations by aCGH. To apply the aCGH technique to the diagnosis as well as investigation of multiple congenital anomalies and mental retardation (MCA/MR), we constructed a consortium with 23 medical institutes and hospitals in Japan, and recruited 536 patients with clinically uncharacterized MCA/MR, whose karyotypes were normal according to conventional cytogenetics, for two-stage screening using two types of bacterial artificial chromosome-based microarray. The first screening using a targeted array detected pCNV in 54 of 536 cases (10.1%), whereas the second screening of the 349 cases negative in the first screening using a genome-wide high-density array at intervals of approximately 0.7 Mb detected pCNVs in 48 cases (13.8%), including pCNVs relevant to recently established microdeletion or microduplication syndromes, CNVs containing pathogenic genes and recurrent CNVs containing the same region among different patients. The results show the efficient application of aCGH in the clinical setting.
Journal of Human Genetics (2011) **56,** 110–124; doi:10.1038/jhg.2010.129; published online 28 October 2010

Keywords: array-CGH; congenital anomaly; mental retardation; screening

INTRODUCTION

Mental retardation (MR) or developmental delay is estimated to affect 2–3% of the population.[1] However, in a significant proportion of cases, the etiology remains uncertain. Hunter[2] reviewed 411 clinical cases of MR and reported that a specific genetic/syndrome diagnosis was carried out in 19.9% of them. Patients with MR often have congenital anomalies, and more than three minor anomalies can be useful in the diagnosis of syndromic MR.[2,3] Although chromosomal aberrations are well-known causes of MR, their frequency determined by conventional karyotyping has been reported to range from 7.9 to 36% in patients with MR.[4–8] Although the diagnostic yield depends on the population of each study or clinical conditions, such studies

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Received 20 August 2010; revised 25 September 2010; accepted 30 September 2010; published online 28 October 2010

suggest that at least three quarters of patients with MR are undiagnosed by clinical dysmorphic features and karyotyping.

In the past two decades, a number of rapidly developed cytogenetic and molecular approaches have been applied to the screening or diagnosis of various congenital disorders including MR, congenital anomalies, recurrent abortion and cancer pathogenesis. Among them, array-based comparative genome hybridization (aCGH) is used to detect copy-number changes rapidly in a genome-wide manner and with high resolution. The target and resolution of aCGH depend on the type and/or design of mounted probes, and many types of microarray have been used for the screening of patients with MR and other congenital disorders: bacterial artificial chromosome (BAC)-based arrays covering whole genomes,[9,10] BAC arrays covering chromosome X,[11,12] a BAC array covering all subtelomeric regions,[13] oligonucleotide arrays covering whole genomes,[14,15] an oligonucleotide array for clinical diagnosis[16] and a single nucleotide polymorphism array covering the whole genome.[17] Because genome-wide aCGH has led to an appreciation of widespread copy-number variants (CNVs) not only in affected patients but also in healthy populations,[18–20] clinical cytogenetists need to discriminate between CNVs likely to be pathogenic (pathogenic CNVs, pCNVs) and CNVs less likely to be relevant to a patient's clinical phenotypes (benign CNVs, bCNVs).[21] The detection of more CNVs along with higher-resolution microarrays needs more chances to assess detected CNVs, resulting in more confusion in a clinical setting.

We have applied aCGH to the diagnosis and investigation of patients with multiple congenital anomalies and MR (MCA/MR) of unknown etiology. We constructed a consortium with 23 medical institutes and hospitals in Japan, and recruited 536 clinically uncharacterized patients with a normal karyotype in conventional cytogenetic tests. Two-stage screening of copy-number changes was performed using two types of BAC-based microarray. The first screening was performed by a targeted array and the second screening was performed by an array covering the whole genome. In this study, we diagnosed well-known genomic disorders effectively in the first screening, assessed the pathogenicity of detected CNVs to investigate an etiology in the second screening and discussed the clinical significance of aCGH in the screening of congenital disorders.

MATERIALS AND METHODS

Subjects

We constructed a consortium of 23 medical institutes and hospitals in Japan, and recruited 536 Japanese patients with MCA/MR of unknown etiology from July 2005 to January 2010. All the patients were physically examined by an expert in medical genetics or a dysmorphologist. All showed a normal karyotype by conventional approximately 400–550 bands-level G-banding karyotyping. Genomic DNA and metaphase chromosomes were prepared from peripheral blood lymphocytes using standard methods. Genomic DNA from a lymphoblastoid cell line of one healthy man and one healthy woman were used as a normal control for male and female cases, respectively. All samples were obtained with prior written informed consent from the parents and approval by the local ethics committee and all the institutions involved in this project. For subjects in whom CNV was detected in the first or second screening, we tried to analyze their parents as many as possible using aCGH or fluorescence *in situ* hybridization (FISH).

Array-CGH analysis

Among our recently constructed in-house BAC-based arrays,[22] we used two arrays for this two-stage survey. In the first screening we applied a targeting array, 'MCG Genome Disorder Array' (GDA). Initially GDA version 2, which contains 550 BACs corresponding to subtelomeric regions of all chromosomes except 13p, 14p, 15p, 21p and 22p and causative regions of about 30 diseases already reported, was applied for 396 cases and then GDA version 3, which contains 660 BACs corresponding to those of GDA version 2 and pericentromeric regions of all chromosomes, was applied for 140 cases. This means that a CNV detected by GDA is certainly relevant to the patient's phenotypes. Subsequently in the second screening we applied 'MCG Whole Genome Array-4500' (WGA-4500) that covers all 24 human chromosomes with 4523 BACs at intervals of approximately 0.7 Mb to analyze subjects in whom no CNV was detected in the first screening. WGA-4500 contains no BACs spotted on GDA. If necessary, we also used 'MCG X-tiling array' (X-array) containing 1001 BAC/PACs throughout X chromosome other than pseudoautosomal regions.[12] The array-CGH analysis was performed as previously described.[12,23]

For several subjects we applied an oligonucleotide array (Agilent Human Genome CGH Microarray 244K; Agilent Technologies, Santa Clara, CA, USA) to confirm the boundaries of CNV identified by our in-house BAC arrays. DNA labeling, hybridization and washing of the array were performed according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner (G2565BA), and the CGH Analytics program version 3.4.40 (Agilent Technologies) was used to analyze copy-number alterations after data extraction, filtering and normalization by Feature Extraction software (Agilent Technologies).

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed as described elsewhere[23] using BACs located around the region of interest as probes.

RESULTS

CNVs detected in the first screening

In the first screening, of 536 cases subjected to our GDA analysis, 54 (10.1%) were determined to have CNV (Figure 1; Tables 1 and 2).

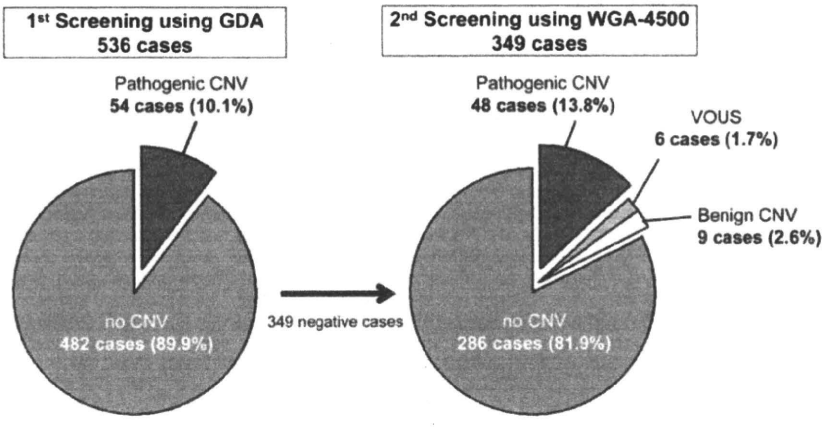

Figure 1 Percentages of each screening in the current study.

Table 1 A total of 40 cases with CNV at subtelomeric region(s) among 54 positive cases in the first screening

	Position where CNV detected				
Gender	*Loss*	*Gain*	*Corresponding disorder*[a]	*OMIM or citation*	*Parental analysis*[b]
M	1p36.33		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1q44		Chromosome 1q43-q44 deletion syndrome	#612337	
F	2q37.3		2q37 monosomy[c]	Shrimpton *et al.*[24]	
F	2q37.3		2q37 monosomy[c]	Shrimpton *et al.*[24]	
M	3q29		Chromosome 3q29 deletion syndrome	#609425	
F	5p15.33p15.32		Cri-du-chat syndrome	#123450	
M	5q35.2q35.3		Chromosome 5q subtelomeric deletion syndrome	Rauch *et al.*[25]	
F	6p25.3		Chromosome 6pter-p24 deletion syndrome	#612582	
M	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	
F	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	
M	9p24.3p24.2		Chromosome 9p deletion syndrome	#158170	
F	9q34.3		Kleefstra syndrome	#610253	
F	10q26.3		Chromosome 10q26 deletion syndrome	#609625	
F	16p13.3		Chromosome 16p13.3 deletion syndrome	#610543	
F	22q13.31		Chromosome 22q13 deletion syndrome	#606232	
M	22q13.31q13.33		Chromosome 22q13 deletion syndrome	#606232	
M		15q26.3	15q overgrowth syndrome[c]	Tatton-Brown *et al.*[27]	
F		15q26.3	15q overgrowth syndrome[c]	Tatton-Brown *et al.*[27]	
M		21q22.13q22.3	Down's syndrome (partial trisomy 21)	#190685	
M		Xp22.33	A few cases have been reported; e.g. V5-130 in Lu *et al.*[28]		
M		Xq28	Chromosome Xq28 duplication syndrome	#300815	
F	1q44		Chromosome 1q43-q44 deletion syndrome	#612337	
		8p23.2p23.3			
M	3p26.3		3p deletion syndrome[d]	Fernandez *et al.*[29]	
		12p13.33p11.22			
F	3p26.3		3p deletion syndrome[d]	Fernandez *et al.*[29]	
		16p13.3	Chromosome 16p13.3 duplication syndrome	#613458	
F	4q35.2		4q− syndrome[d]	Jones *et al.*[30]	
		7q36.3			
M	5p15.33		Cri-du-chat syndrome	#123450	
		20p13			
M	5p15.33p15.32		Cri-du-chat syndrome	#123450	
		2p25.3			
F	6q27		6q terminal deletion syndrome[d]	Striano *et al.*[31]	
		11q25			
F	6q27		6q terminal deletion syndrome[d]	Striano *et al.*[31]	
		8q24.3			
M	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	*dn*
		1q44			
M	9p24.3p24.2		Chromosome 9p deletion syndrome	#158170	
		7q36.3			
F	10p15.3p15.2		Chromosome 10p terminal deletion[d]	Lindstrand *et al.*[32]	*pat*
		7p22.3p22.2			
M	10p15.3		Chromosome 10p terminal deletion[d]	Lindstrand *et al.*[32]	
		2p25.3			
M	10q26.3		Chromosome 10q26 deletion syndrome	#609625	
		2q37.3	Distal trisomy 2q[d]	Elbracht *et al.*[33]	
M	18q23		Chromosome 18q deletion syndrome	#601808	
		7q36.3			
F	22q13.31q13.33		Chromosome 22q13.3 deletion syndrome	#606232	*pat*
		17q25.3	One case was reported	Lukusa *et al.*[34]	
M	Xp22.33/Yp11.32		Contiguous gene–deletion syndrome on Xp22.3[d]	Fukami *et al.*[35]	
		Xq27.3q28	Chromosome Xq28 duplication syndrome	#300815	

Abbreviations: F, female; CNV, copy-number variant; M, male; OMIM, Online Mendelian Inheritance in Man; *dn*, *de novo* CNV observed in neither of the parents.
[a]The name of disorder is based on entry names of OMIM, expect for entry names in DECIPHER and description in each cited article.
[b]*pat*, father had a balanced translocation involved in corresponding subtelomeric regions.
[c]Entry names in DECIPHER.
[d]Description in each cited article.

All the CNVs detected in the first screening were confirmed by FISH. Among the positive cases, in 24 cases one CNV was detected. All the CNVs corresponded to well-established syndromes or already described disorders (Table 1). In 16 cases two CNVs, one deletion and one duplication, were detected at two subtelomeric regions, indicating that one of parents might be a carrier with reciprocal translocation involved in corresponding subtelomeric regions, and at least either of the two CNVs corresponded to the disorders. We also performed parental analysis by FISH for three cases whose parental samples were available, and confirmed that in two cases the subtelomeric aberrations were inherited from paternal balanced translocation and in one case the subtelomeric aberrations were *de novo* (Table 1). In the other 14 cases, CNVs (25.9%) were detected in regions corresponding to known disorders (Table 2).

Table 2 Other cases among 54 positive cases in the first screening

	Position where CNV detected			
Gender	*Gain*	*Loss*	*Corresponding disorder*	*OMIM*
F		4p16.3	Ring chromosome	
		4q35.2		
M		3q22.323	BPES	#110100
M		2q22.3	ZFHX1B region	*605802
M		4q22.1	Synuclein (SNCA) region	*163890
F		7p21.1	Craniosynostosis, type 1	#123100
F		7q11.23	Williams syndrome	#194050
F		8q23.3q24.11	Langer–Giedion syndrome	#150230
M	15q11.2q13.1		Prader–Willi/Angelman	#176270/ #105830
F		17p11.2	Smith–Magenis syndrome	#182290
M		17q11.2	Neurofibromatosis, type I	+162200
M	22q11.21		DiGeorge syndrome	#188400
F		22q11.21	DiGeorge syndrome	#188400
F	Xp22.31		Kallmann syndrome 1	+308700
F	Whole X		Mosaicism	

Abbreviations: CNV, copy-number variant; F, female; M, male; OMIM, Online Mendelian Inheritance in Man.

CNVs detected in the second screening and assessment of the CNVs

Cases were subject to the second screening in the order of subjects detected no CNV in the first screening, and until now we have analyzed 349 of 482 negative cases in the first screening. In advance, we excluded highly frequent CNVs observed in healthy individuals and/or in multiple patients showing disparate phenotypes from the present results based on an internal database, which contained all results of aCGH analysis we have performed using WGA-4500, or other available online databases; for example, Database of Genomic Variant (http://projects.tcag.ca/variation/). As a result, we detected 66 CNVs in 63 cases (Figure 1; Table 3). Among them, three patients (cases 36, 42 and 44) showed two CNVs. All the CNVs detected in the second screening were confirmed by other cytogenetic methods including FISH and/or X-array. For 60 cases, we performed FISH for confirmation and to determine the size of each CNV. For five cases, cases 13, 36, 48, 57 and 63, with CNVs on the X chromosome, we used the X-array instead of FISH. For cases 4, 6, 16–19 and 34, we also used Agilent Human Genome CGH Microarray 244K to determine the refined sizes of CNVs. The maximum and minimum sizes of each CNV determined by these analyses are described in Table 3.

Well-documented pCNVs emerged in the second screening

CNVs identified for recently established syndromes. We assessed the pathogenicity of the detected CNVs in several aspects (Figure 2).[21,37,38] First, in nine cases, we identified well-documented pCNVs, which are responsible for syndromes recently established. A heterozygous deletion at 1q41–q42.11 in case 2 was identical to patients in the first report of 1q41q42 microdeletion syndrome.[39] Likewise a CNV in case 3 was identical to chromosome 1q43–q44 deletion syndrome (OMIM: #612337),[40] a CNV in case 4 was identical to 2q23.1 microdeletion syndrome,[41] a CNV in case 5 was identical to 14q12 microdeletion syndrome[42] and a CNV in case 6 was identical to chromosome 15q26-qter deletion syndrome (Drayer's syndrome) (OMIM: #612626).[43] Cases 7, 8 and 9 involved CNVs of different sizes at 16p12.1-p11.2, the region responsible for 16p11.2-p12.2 microdeletion syndrome.[44,45] Although an interstitial deletion at 1p36.23-p36.22 observed in case 1 partially overlapped with a causative region of chromosome 1p36 deletion syndrome (OMIM: #607872), the region deleted was identical to a proximal interstitial 1p36 deletion that was recently reported.[46] Because patients with the proximal 1p36 deletion including case 1 demonstrated different clinical characteristics from cases of typical chromosome 1p36 deletion syndrome, in the near term their clinical features should be redefined as an independent syndrome.[46]

CNVs containing pathogenic gene(s). In four cases we identified pCNVs that contained a gene(s) probably responsible for phenotypes. In case 10, the CNV had a deletion harboring *GLI3* (OMIM: *165240) accounting for Greig cephalopolysyndactyly syndrome (GCS; OMIM: 175700).[47] Although phenotypes of the patient, for example, pre-axial polydactyly of the hands and feet, were consistent with GCS, his severe and atypical features of GCS, for example, MR or microcephaly, might be affected by other contiguous genes contained in the deletion.[48] Heterozygous deletions of *BMP4* (OMIM: *112262) in case 11 and *CASK* (OMIM: *300172) in case 13 have been reported previously.[49,50] In case 12, the CNV contained *YWHAE* (OMIM: *605066) whose haploinsufficiency would be involved in MR and mild CNS dysmorphology of the patient because a previous report demonstrated that haploinsufficiency of *ywhae* caused a defect of neuronal migration in mice[51] and a recent report also described a microdeletion of *YWHAE* in a patient with brain malformation.[52]

Recurrent CNVs in the same regions. We also considered recurrent CNVs in the same region as pathogenic; three pairs of patients had overlapping CNVs, which have never been reported previously. Case 16 had a 3.3-Mb heterozygous deletion at 10q24.31–q25.1 and case 17 had a 2.0-Mb deletion at 10q24.32–q25.1. The clinical and genetic information will be reported elsewhere. Likewise, cases 14 and 15 also had an overlapping CNV at 6q12–q14.1 and 6q14.1, and cases 18 and 19 had an overlapping CNV at 10p12.1–p11.23. Hereafter, more additional cases with the recurrent CNV would assist in defining new syndromes.

CNVs reported as pathogenic in previous studies. Five cases were applicable to these criteria. A deletion at 3p21.2 in case 20 overlapped with that in one case recently reported.[53] The following four cases had CNVs reported as pathogenic in recent studies: a CNV at 7p22.1 in case 21 overlapped with that of patient 6545 in a study by Friedman *et al.*,[14] a CNV at 14q11.2 in case 22 overlapped with those of patients 8326 and 5566 in Friedman *et al.*,[14] a CNV at 17q24.1–q24.2 in case 23 overlapped with that in patient 99 in Buysse *et al.*[54] and a CNV at 19p13.2 in case 24 overlapped with case P11 in Fan *et al.*[55]

Large or gene-rich CNVs, or CNVs containing morbid OMIM genes. In cases inapplicable to the above criteria, we assessed CNVs

Table 3 Sixty-three cases with CNV in the 2nd screening

Case	Gender	Clinical diagnosis	Remarkable clinical features	CNV	Position	WGA-4500[b]	FISH[b]	Base position and size of the identified CNV[a]					Parental analysis	Protein-coding genes[c]	CNV assessment[d]	Corresponding or candidate gene(s)
								Start (max)	Start (min)	End (min)	End (max)	Size (min)	Size (max)			
1	M	MCA/MR		del	1p36.23p36.22	arr cgh 1p36.23p36.22 (RP11-81J7→RP11-199O1)x1	ish del(1)(p36.23p36.22)(RP11-462M3+,RP11-106A3-,RP11-28P4+)dn	8 585 127	8 890 860	10 561 097	11 143 717	1 670 237	2 558 590	*dn*	32	P	
2	M	MCA/MR		del	1q41q42.11	arr cgh 1q41 (RP11-135J2→RP11-239E10)x1	ish del(1)(q41q42.11)(RP11-706L9+,RP11-224O19-,RP11-36704-)dn	215 986 492	216 532 600	221 534 398	222 467 931	5 001 798	6 481 439	*dn*	35	P	
3	F	MCA/MR	Epilepsy	del	1q44	arr cgh 1q44 (RP11-156E8)x1	ish del(1)(q44)(RP11-56O19+,RP11-156E8-)	241 996 973	243 177 632	243 251 660	244 141 010	74 028	2 144 037		11	P	
4	F	MCA/MR		del	2q22	arr cgh 2q23.1 (RP11-72H23)x1	ish del(2)(q23.1)(RP11-375H16-)	147 651 472	147 688 255	149 855 826	149 879 891	2 167 571	2 228 419		7	P	
5	F	MCA/MR		del	14q12q13.2	arr cgh 14q12q13.2 (RP11-36909→RP11-26M6)x1	ish del(14)(q13.2)(RP11-831F6-)	28 768 137	29 297 829	34 689 412	35 489 337	5 391 583	6 721 200		25	P	
6	M	MCA/MR	CHD	del	15q26.2	arr cgh 15q26.2q26.3 (RP11-79C10→RP11-80F4)x1	ish del(15)(q26.2)(RP11-308P12-)	93 199 415	93 214 053	96 928 421	96 942 334	3 714 368	3 742 919		6	P	
7	M	MCA/MR	CHD	del	16p12.1p11.2	arr cgh 16p12.1p11.2 (RP11-309I14→RP11-150K5)x1	ish del(16)(p11.2)(RP11-75J11-)dn	25 795 340	27 008 538	29 825 404	31 443 492	2 816 866	5 648 152	*dn*	138	P	
8	M	MCA/MR	CHD	del	16p11.2	arr cgh 16p12.1p11.2 (RP11-360L15→RP11-150K5)x1	ish del(16)(p11.2)(RP11-360L15-,RP11-388M20+,RP11-75J11+)dn	27 184 508	28 873 631	29 825 404	31 443 492	951 773	4 258 984	*dn*	134	P	
9	F	MCA/MR		del	16p11.2	arr cgh 16p11.2 (RP11-368N21→RP11-499D5)x1	ish del(16)(p11.2)(RP11-388M20-,RP11-75J11-)	28 873 841	29 408 698	32 773 200	34 476 095	3 364 502	5 602 254		125	P	
10	M	MCA/MR		del	7p14.2p13	arr cgh 7p14.2p13 (RP11-138E20→RP11-52M17)x1	ish del(7)(p14.1p13)(RP11-258I11+,RP11-2J17-,RP11-346F12-)dn	35 621 006	36 470 190	44 657 334	45 508 196	8 187 144	9 887 190	*dn*	70	P	*GLI3*
11	F	MCA/MR	Corneal opacity	del	14q22.1q22.3	arr cgh 14q22.1q22.3 (RP11-122A4→RP11-172G1)x1	ish del(14)(q22.1)(RP11-122A4-,RP11-316L15+)dn	51 964 774	51 983 834	54 730 496	55 054 754	2 746 662	3 089 980	*dn*	18	P	*BMP4*
12	M	MCA/MR	Idiopathic leukocystrophy	del	17q13.3	arr cgh 17p13.3 (RP11-294I5→RP11-357O7)x1	ish del(17)(p13.3)(RP11-4F24-,RP11-26N6+)dn	1 008 128	1 146 211	2 077 151	2 026 967	930 940	1 018 839	*dn*	22	P	*YWHAE*
13	M	MCA/MR		del	Xp11.4p11.3	arr cgh Xp11.3p11.4 (RP11-1069J5→RP11-245M24)x1	ish del(X)(p11.4p11.3)(RP11-95C16-,RP11-829C10-)dn	41 392 291	41 385 453	45 419 624	45 495 709	4 034 171	4 103 418	*dn*	9	P	*CASK*

Table 3 Continued

Case	Gender	Clinical diagnosis	Remarkable clinical features	CNV	Position	WGA-4500[b]	FISH[b]	Base position and size of the identified CNV[a]						Parental analysis	Protein-coding genes[c]	CNV assessment[d]	Corresponding or candidate gene(s)
								Start (max)	Start (min)	End (min)	End (max)	Size (min)	Size (max)				
14	M	MCA/MR		del	6q12q14.1	arr cgh 6q12q14.2(RP11-502L6→RP11-232L4)x1	ish del(6)(q13)(RP11-28P18-)dn	69029871	69731888	83926178	85101718	14194290	16071847	dn	56	P	
15	M	ZLS		del	6q14.1	arr cgh 6q14.1 (RP11-343P23→RP11-217L13)x1	ish del(6)(q14.1) (RP11-5N7-,RP11-990K4-,RP11-116+)	75484004	76145436	79474428	79851528	3328992	4367524		10	P	
16	F	MCA/MR	CHD	del	10p12.1p11.23	arr cgh 10p12.1p11.23 (RP11-89D1→91A23)x1	ish del(10)(p12.1p11.23) (RP11-164A7-,RP11-110B21-)	27045285	27054002	29057401	29088950	2003399	2043665		18	P	
17	M	MCA/MR		del	10p12.1p11.23	arr cgh 10p12.1p11.23 (RP11-218D6→RP11-RP11-181I11)x1	ish del(10)(p11.23) (RP11-15H10-)	28121596	28131608	30559024	30577807	2427416	2456211		12	P	
18	M	MCA/MR	CHD	del	10q24.31q25.1	arr cgh 10q24.31q25.1 (RP11-108L7→RP11-108L7)x1	ish del(10)(q24.33) (RP11-416N2-)dn	102560783	102568462	105914057	105929608	3345595	3368825	dn	66	P	
19	M	MCA/MR		del	10q24.32q25.1	arr cgh 10q24.32q25.1 (RP11-21N23→RP11-99N20)x1	ish del(10)(q24.33) (RP11-416N2-)dn	103917900	103928189	106005827	106011522	2077638	2093622	dn	41	P	
20	F	MCA/MR		del	3p21.31p21.2	arr cgh 3p21.31p21.2 (RP11-24F11→RP11-89F17)x1	ish del(3)(p21.31) (RP11-3B7-)	46150261	46359965	51390597	52571544	5030632	6421283		175	P	
21	M	MCA/MR		del	7p22.1	arr cgh 7p22.1 (RP11-90J23→RP11-2K20)x1	ish del(7)(p22.1) (RP11-2K20-)dn	3185609	5892225	6233987	6409277	341762	3223668	dn	28	P	
22	F	MCA/MR	Corneal opacity, CHD	dup	14q11.2	arr cgh 14q11.2 (RP11-152G22→RP11-84D12)x3	ish dup(14)(q11.2) (RP11-152G22++)	20070731	20306624	20534929	21264945	228305	1194214		>30	P	
23	M	MCA/MR		del	17q24.1q24.2	arr cgh 17q24.1q24.2 (RP11-89L7→RP11-79K13)x1	ish del(17)(q24.1q24.2) (RP11-93E5-,RP11-89L7-,RP11-79K13-)	60576365	60936391	64592701	64587782	3656310	4011417		29	P	
24	M	SMS susp.		del	19p13.2	arr cgh 19p13.2 (RP11-19704→RP11-164D24)x1	ish del(19)(p13.2) (91021-)	9248377	10248853	11968772	12553279	1719919	3304902	dn		P	
25	M	MCA/MR	Epilepsy	dup	2q11.2q13	arr cgh 2q11.2q13(RP11-90G13→RP11-79K7)x3	ish dup(2)(q11.2) (RP11-542D13++)	88273220	91696986	109869691	112714666	18172705	24441446		>30	P	
26	M	MCA/MR	CHD	dup	4p16.1	arr cgh 4p16.1 (RP11-17I9)x3	ish dup(4)(p16.1) (RP11-301J10++)	8202790	8520479	9793705	10638054	1273226	2435264		17	P	

Table 3 Continued

Case	Gender	Clinical diagnosis	Remarkable clinical features	CNV	Position	WGA-4500[b]	FISH[b]	Base position and size of the identified CNV[a]						Parental analysis	Protein-coding genes[c]	CNV assessment[d]	Corresponding or candidate gene(s)
								Start (max)	Start (min)	End (min)	End (max)	Size (min)	Size (max)				
27	F	MCA/MR		del	7q22.1q22.2	arr cgh 7q22.1q22.2 (RP11-10D8→ RP11-72J24)x1	ish del(7)(q22.1q22.2) (RP11-124G15+,RP11-188E1-,RP11-95P19-)	97314215	98261079	105604920	106451506	7343841	9137291		135	P	
28	F	MCA/MR	Epilepsy	del	12q13.13	arr cgh 12q13.13 (RP11-74I8→ RP11-624J6)x1	ish del(12)(q13.13) (RP11-624J6-)	50987232	51016427	51956291	52180088	939864	1192856		44	P	
29	M	MCA/MR		dup	16q22.3	arr cgh 16q22.3 (RP11-90L19→ RP11-89K4)x3	ish dup(16)(q22.3) (RP11-115E3++, RP11-90L19++)	70355260	70848592	72328913	73785124	1480321	3429864		25	P	
30	M	RTS susp.		dup	16q24.1	arr cgh 16q24.1 (RP11-140K16→ RP11-442O1)x3	ish dup(16)(q24.1) (RP11-770B4++, RP11-140K16++)	82699729	82797548	83749375	84123857	951827	1424128		16	P	
31	M	MCA/MR	Epilepsy	del	2q24.2q24.3	arr cgh 2q24.2 (RP11-89L13→ RP11-79L13)x1	ish del(2)(q24.2) (RP11-638N12-)	160407234	161072815	162883584	166923475	1810769	6516241		28	P	*TBR1*
32	M	MCA/MR		del	3p26.2	arr cgh 3p26.2 (RP11-32F23)x1	ish del(3)(p26.2) (RP11-32F23-)	3943353	4016797	4198468	4329970	181671	386617		2	P	*SUMF1*
33	M	MCA/MR	IgA deficiency	del	7q21.11	arr cgh 7q21.11 (RP11-22M18)x1	ish del(7)(q21.11) (RP11-115M2+, RP11-353O4-, RP11-22M18-)	83597839	83601541	84549609	84788160	948068	1190321		3	P	*SEMA3A*
34	M	MCA/MR		dup	14q32.2	arr cgh 14q32.2 (RP11-128L1)x3	ish dup(14)(q32.2) (RP11-177F8++)	99330486	99337358	99841558	99845472	504200	514986		7	P	*EML1, YY1*
35	M	MCA/MR	Epilepsy	dup	16p13.3	arr cgh 16p13.3 (RP11-349I11)x3	ish dup(16)(p13.3) (RP11-349I11++)	4851459	5678447	5906909	6165923	228462	1314464		9	P	*A2BP1*
36	M	MCA/MR		dup	Xp22.2p22.13	arr cgh Xp22.2p22.13 (RP11-2K15→ RP11-115I10)x3	not performed (X-tiling array)	16874735	16952121	17596600	17638351	644479	763616		2	P	
				dup	Xp21.3	arr cgh Xp21.3 (RP11-438J7)x3	not performed (X-tiling array)	28704076	28704076	28868075	28868075	163999	163999		1	P	*IL1RAPL1*
37	F	MCA/MR		del	1p34.3	arr cgh 1p34.3 (RP11-89N10→ RP11-416A14)x1	ish del(1)(p34.2) (RP11-195A8+, RP11-166F21-)dn	37830131	38338265	39466349	39583645	1128084	1753514	*dn*	7	P	
38	M	MCA/MR	Hyper IgE	dup	1q25.2	arr cgh 1q25.2 (RP11-177A2→ RP11-152A16)x3	ish dup(1)(1q25.2) (RP11-177A2++, RP11-152A16++)	177088480	177196858	177535659	177859828	338801	771348	*dn*	9	P	
39	M	MCA/MR		del	2p24.1p23.3	arr cgh 2p24.1p23.3 (RP11-80H16→ RP11-88F6)x1	ish del(2)(p23.3) (RP11-88F6-, RP11-373D23+)dn	20037821	23094244	26815794	28414457	3721550	8376636	*dn*	86	P	
40	F	MCA/MR	CHD	del	3p26.1p25.3	arr cgh 3p26.1p25.3 (RP11-128A5→ RP11-402P11)x1	ish del(3)(p26.1p25.3) (RP11-936E1-, RP11-402P11-, RP11-1079H21+) dn	8190557	8497949	9930973	10026217	1433024	1835660	*dn*	18	P	

Table 3 Continued

Case	Gender	Clinical diagnosis	Remarkable clinical features	CNV	Position	WGA-4500[b]	FISH[b]	Base position and size of the identified CNV[a]						Parental analysis	Protein-coding genes[c]	CNV assessment[d]	Corresponding or candidate gene(s)
								Start (max)	Start (min)	End (min)	End (max)	Size (min)	Size (max)				
41	M	MCA/MR		del	3p22.1p21.31	arr cgh 3p22.1p21.31 (RP11-241P3→ RP11-88B8)x1	ish del(3)(p22.1) (RP11-61H16+, RP11-241P3-, RP11-78O10+)dn	41 365 663	42 284 365	48 177 538	49 198 542	5 893 173	7 832 879	*dn*	123	P	
42	M	MCA/MR	Corneal opacity	del	3p14.3p14.2	arr cgh 3p14.3p14.2 (RP11-80H18→ RP11-79J9)x1	ish del(3)(p14.2) (RP11-79J19-, RP11-230A22+)mat	57 370 434	58 149 199	58 742 633	58 887 574	593 434	1 517 140	*mat*	11	B	
				del	8q21.11q21.13	arr cgh 8q21.11q21.13 (RP11-225J6→ RP11-214E11)x1	ish del(8) (q21.11q21.13) (RP11-225J6-, RP11-48B3+)dn	75 722 961	75 821 163	81 110 557	81 493 446	5 289 394	5 770 485	*dn*	12	P	
43	M	MCA/MR		del	3q26.31q26.33	arr cgh 3q26.31-q26.33 (RP11-292L5→ RP11-355N16)x1	ish del(3)(q26.32) (RP11-300L9+, RP11-105L6-)dn	175 650 310	176 531 688	180 613 203	181 653 281	4 081 515	6 002 971	*dn*	12	P	
44	M	MCA/MR	CHD	del	13q13.2q13.3	arr cgh 13q13.2 (RP11-269G10→ 90F5)x1	ish del(13)(q13.2) (RP11-142E9+, RP11-381E21-, RP11-98D3+)dn	33 451 136	33 895 560	34 813 379	34 909 905	917 819	1 458 769	*dn*	1	P	
				del	22q11.21	arr cgh 22q11.21 (RP11-155F20→ 54C2)x1	ish del(22)(q11.21) (RP11-155F20-, RP11-590C5-, RP11-54C2-)pat	19 310 307	19 310 307	19 590 642	19 590 642	280 335	280 335	*pat*	15	B	
45	F	aRS		del	18q21.2	arr cgh 18q21.2 (RP11-89B14)x1	ish del(18)(q21.2) (RP11-159D14+, RP11-186B13-, RP11-111C17-)dn	48 218 621	49 166 752	51 288 665	51 861 143	2 121 913	3 642 522	*dn*	9	P	
46	M	MCA/MR		dup	19p13.3	arr cgh 19p13.3 (RP11-49M3→ RP11-268O21)x3		1 095 485	2 418 857	3 499 581	4 460 252	1 080 724	3 364 767	*dn*	113	P	
47	F	MCA/MR	Autism	del	19p13.3	arr cgh 19p13.3 (RP11-30F17→ RP11-330I7)x1	ish del(19)(p13.3) (RP11-330I7-)dn	4 844 383	6 043 505	6 859 584	6 881 792	816 079	2 037 409	*dn*	23	P	
48	M	MCA/MR		del	Xp11.3	arr cgh Xp11.3 (RP11-151G3→ RP11-48J14)x0	ish del(X)(p11.3) (RP11-203D16-)mat	44 403 077	44 433 162	46 795 584	46 795 588	2 362 422	2 392 511	*mat*	18	P	
49	M	MCA/MR		dup	3p26.3	arr cgh 3p26.3 (RP11-630I)x3	ish dup(3)(p26.3) (RP11-630I++)pat	2 377 366	2 443 357	2 619 407	2 628 216	176 050	250 850	*pat*	1	B	
50	M	MCA/MR		dup	5p14.3	arr cgh 5p14.3 (RP11-91A5)x3	ish dup(5)(p14.3) (RP11-91A5++)pat	19 046 234	19 485 530	19 656 108	20 798 445	170 578	1 752 211	*pat*	1	B	
51	M	MCA/MR		dup	5q13.3	arr cgh 5q13.1 (RP11-40N8→ RP11-91C10)x3	ish dup(5)(q13.1) (RP11-105A11++)mat	66 417 271	66 481 371	67 501 700	67 838 977	1 020 329	1 421 706	*mat*	3	B	

Table 3 Continued

Case	Gender	Clinical diagnosis	Remarkable clinical features	CNV Position	WGA-4500[b]	FISH[b]	Base position and size of the identified CNV[a]						Parental analysis	Protein-coding genes[c]	CNV assessment[d]	Corresponding or candidate gene(s)
							Start (max)	Start (min)	End (min)	End (max)	Size (min)	Size (max)				
52	M	MCA/MR		dup 7p22.3	arr cgh 7p22.3 (RP11-23D23)x3	ish dup(7)(p22.3) (RP11-23D23++, RP11-1133D5+)mat	1	954016	954584	1101944	568	1101943	*mat*	12	B	
53	F	MCA/MR		dup 8p23.2	arr cgh 8p23.2 (RP11-79I19→ RP11-89I12)x3	ish dup(8)(p23.2) (RP11-89I19++, RP11-89I12++)pat	3324954	3726061	4564671	5973493	838610	2648539	*pat*	1	B	
54	M	MCA/MR		dup 9q33.1	arr cgh 9q33.1 (RP11-150L1)x3	ish dup(9)(q33.1) (RP11-150L1++)pat	118980752	119452372	119614984	120011559	162612	1030807	*pat*	2	B	
55	F	MCA/MR		dup 10q22.3	arr cgh 10q22.3 (RP11-79M9)x3	ish dup(10)(q22.3) (RP11-79M9++)mat	77356915	77718484	77873148	78230039	154664	873124	*mat*	1	B	
56	M	MCA/MR	ELBW, hepato-blastoma	dup 12q21.31	arr cgh 12q21.31 (RP11-91C4)x3	ish dup(12)(q21.31) (RP11-91C4++, RP11-142L2+)pat	80924954	82678148	82830190	85768388	152042	4843434	*pat*	3	B	
57	M	GS		del Xp11.23	arr cgh Xp11.23 (RP11-876B24) x0 mat	not performed (X-tiling array)	47752808	47747918	47852109	47868412	104191	115604	*mat*	3	B	
58	M	MCA/MR		dup 8q11.23	arr cgh 8q11.23 (RP11-221P7)x3	ish dup(8)(q11.23) (RP11-221P7++, RP11-26P22++)	53665974	53717675	54235229	54576654	517554	910680		3	VOUS	
59	F	MCA/MR	Micro-cephaly	dup 10q11.21	arr cgh 10q11.21 (RP11-178A10)x3	ish dup(10)(q11.21) (RP11-178A10++)	41986946	42197693	42320775	43603027	123082	1616081		15	VOUS	
60	M	MCA/MR		dup 11p14.2p14.1	arr cgh 11p14.2p14.1 (RP11-1L12)x3	ish dup(11) (p14.2p14.1) (RP11-1L12++)	26723462	27033270	27213374	27445504	180104	722042		4	VOUS	
61	F	MCA/MR		dup 12p11.1	arr cgh 12p11.1 (RP11-88P4)x3	ish dup(12)(p11.1) (RP11-472A10++)	33333493	33359944	33572956	33572956	213012	239463		2	VOUS	
62	F	aRS		dup 12q21.31	arr cgh 12q21.31 (RP11-91I24→ RP11-91C4)x3	ish dup(12)(q21.31) (RP11-91C4++, RP11-142L2++)	79949648	82172368	83968319	85768388	1795951	5818740		12	VOUS	
63	F	MR	Congenital myopathy	dup Xq12	arr cgh Xq12 (RP11-90P17→ RP11-383C12)x3	Not performed (X-tiling array)	66212661	66216353	66921699	66948538	705346	735877		1	VOUS	

Abbreviations: aRS, atypical Rett syndrome; B, benign; CNV, copy-number variant; *dn*: *de novo* CNV observed in neither of the parents; ELBW, extremely low birth weight; FISH, fluorescence *in situ* hybridization; GS, Gillespie syndrome; *mat*: CNV identified also in mother; P, pathogenic; *pat*: CNV identified also in father; RTS, Rubinstein–Taybi syndrome; SMS, Smith–Magenis syndrome; VOUS, variant of uncertain clinical significance; ZLS, Zimmermann–Laband syndrome.
[a]The sizes were estimated by WGA-4500, X-array, FISH or Agilent Human Genome CGH microarray 244K.
[b]The notation systems is based on ISCN2005.[36]
[c]The number of protein-coding genes contained in the respective CNVs.
[d]The result of CNV assessment.

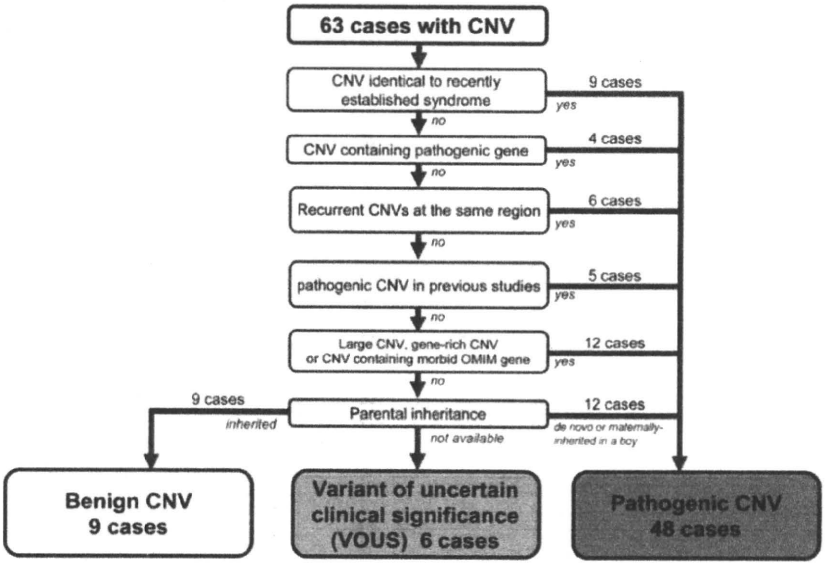

Figure 2 A flowchart of the assessment of CNVs detected in the second screening.

from several aspects. A CNV that contains abundant genes or is large (> 3 Mb) has a high possibility to be pathogenic.[21] The CNVs in cases 25–30 probably correspond to such CNVs. Also, we judged a CNV containing a morbid OMIM gene as pathogenic:[21] *TBR1* (OMIM: *604616) in case 31,[56] *SUMF1* (OMIM: *607939) in case 32,[57,58] *SEMA3A* (OMIM: *603961) in case 33,[59] *EML1* (OMIM: *602033) and/or *YY1* (OMIM: *600013) in case 34,[60,61] *A2BP1* (OMIM: *605104) in case 35[62] and *IL1RAPL1* (OMIM: *300206) in case 36.[63] Several previous reports suggest that these genes are likely to be pathogenic, although at present no evidence of a direct association between these genes and phenotypes exists.

CNVs de novo or X maternally inherited. Among the remaining 27 cases, 12 cases had CNVs considered pathogenic as their CNVs were *de novo* (cases 37–47) or inherited del(X)(p11.3) from the mother (case 48). In the second screening we performed FISH for 36 CNVs of the 34 cases whose parental samples were available to confirm that 24 cases had *de novo* CNVs, which were probably pathogenic. A CNV in case 48, a boy with a nullizygous deletion at Xp11.3 inherited from his mother, was also probably relevant to his phenotype (Tables 3 and 4). Meanwhile, although case 57 was a boy with a deletion at Xp11.23 inherited from his mother, he was clinically diagnosed with Gillespie syndrome (OMIM: #206700) that was reported to show an autosomal dominant or recessive pattern,[64] thus we judged that the deletion was not relevant to his phenotype. As a result, cases 49–57 had only CNVs inherited from one of their parents which are likely to be unrelated to the phenotypes; that is, bCNV (Table 4).

As a result, we estimated that 48 cases among 349 analyzed (13.8%) had pCNV(s) in the second screening (Table 3; Figure 2). The CNVs of the remaining six cases, cases 58–63, were not associated with previously reported pathogenicity and their inheritance could not be evaluated, thus we estimated they were variants of uncertain clinical significance (VOUS).[38]

DISCUSSION

Because aCGH is a high-throughput technique to detect CNVs rapidly and comprehensively, this technique has been commonly used for analyses of patients with MCA and/or MR.[38,65–68] However, recent studies of human genomic variation have uncovered surprising properties of CNV, which covers 3.5–12% of the human genome even in healthy populations.[18–20,69] Thus analyses of patients with uncertain clinical phenotypes need to assess whether the CNV is pathogenic or unrelated to phenotypes.[21] However, such an assessment may diminish the rapidness or convenience of aCGH.

In this study, we evaluated whether our in-house GDA can work well as a diagnostic tool to detect CNVs responsible for well-established syndromes or those involved in subtelomeric aberrations in a clinical setting, and then explored candidate pCNVs in cases without any CNV in the first GDA screening. We recruited 536 cases that had been undiagnosed clinically and studied them in a two-stage screening using aCGH. In the first screening we detected CNVs in 54 cases (10.1%). Among them, 40 cases had CNV(s) at subtelomeric region(s) corresponding to the well-established syndromes or the already described disorders and the other 14 cases had CNVs in the regions corresponding to known disorders. Thus about three quarters of cases had genomic aberrations involved in subtelomeric regions. All the subtelomeric deletions and a part of the subtelomeric duplications corresponded to the disorders, indicating that especially subtelomeric deletions had more clinical significance compared to subtelomeric duplications, although the duplication might result in milder phenotypes and/or function as a modifier of phenotypes.[70] Moreover, parental analysis in three cases with two subtelomeric aberrations revealed that two of them were derived from the parental balanced translocations, indicating that such subtelomeric aberrations were potentially recurrent and parental analyses were worth performing. Recently several similar studies analyzed patients with MCA/MR or developmental delay using a targeted array for subtelomeric regions and/or known genomic disorders and detected clinically relevant CNVs in 4.4–17.1% of the patients.[28,65,70,71] Our detection rate in the first screening was equivalent to these reports. Although such detection rates depend on the type of microarray, patient selection criteria and/or number of subjects, these results suggest that at least 10% of cases with undiagnosed MCA/MR and a normal karyotype would be detectable by targeted array.

Table 4 Parental analysis of 34 cases in the second screening

Case	Gender	Clinical diagnosis	CNV		Size of CNV (bp)		Protein-coding genes	Parental analysis	Pathogenicity
			del/dup	Position	Min.	Max.			
1	M	MCA/MR	del	1p36.23p36.22	1 670 237	2 558 590	32	*de novo*	P
2	M	MCA/MR	del	1q41q42.11	5 001 798	6 481 439	35	*de novo*	P
7	M	MCA/MR	del	16p12.1p11.2	2 816 866	5 648 152	138	*de novo*	P
8	M	MCA/MR with CHD	del	16p11.2	951 773	4 258 984	134	*de novo*	P
10	M	MCA/MR	del	7p14.2p13	8 516 513	9 421 233	70	*de novo*	P
11	F	MCA/MR	del	14q22.1q22.3	2 746 662	3 089 980	18	*de novo*	P
12	M	MCA/MR	del	17q13.3	930 940	1 018 839	22	*de novo*	P
13	M	MCA/MR	del	Xp11.4p11.3	4 034 171	4 103 418	9	*de novo*	P
14	M	MCA/MR	del	6q12q14.1	14 194 290	16 071 847	56	*de novo*	P
18	M	MCA/MR	del	10q24.31q25.1	3 345 595	3 368 825	66	*de novo*	P
19	M	MCA/MR	del	10q24.32q25.1	2 077 638	2 093 622	41	*de novo*	P
21	M	MCA/MR	del	7p22.1	341 762	3 223 668	28	*de novo*	P
24	M	SMS susp.	del	19p13.2	1 719 919	3 304 902	23	*de novo*	P
37	F	MCA/MR	del	1p34.3	1 128 084	1 753 514	7	*de novo*	P
38	M	MCA/MR	dup	1q25.2	338 801	771 348	9	*de novo*	P
39	M	MCA/MR	del	2p24.1p23.3	3 721 550	8 376 636	86	*de novo*	P
40	F	MCA/MR	del	3p26.1p25.3	1 433 024	1 835 660	18	*de novo*	P
41	M	MCA/MR	del	3p22.1p21.31	5 893 173	7 832 879	123	*de novo*	P
42[a]	M	MCA/MR	del	8q21.11q21.13	5 289 394	5 770 485	12	*de novo*	P
42[a]	M	MCA/MR	del	3p14.3p14.2	593 434	1 517 140	11	Maternal	B
43	M	MCA/MR	del	3q26.31q26.33	4 081 515	6 002 971	12	*de novo*	P
44[b]	M	MCA/MR	del	13q13.2q13.3	917 819	1 458 769	1	*de novo*	P
44[b]	M	MCA/MR	del	22q11.21	917 819	1 458 769	15	Paternal	B
45	F	Rett syndrome	del	18q21.2	2 121 913	3 642 522	9	*de novo*	P
46	M	MCA/MR	dup	19p13.3	2 041 395	2 404 096	113	*de novo*	P
47	F	MCA/MR	del	19p13.3	816 079	2 037 409	23	*de novo*	P
48[c]	M	MCA/MR	del	Xp11.3	2 362 422	2 392 511	18	Maternal	P
49	M	MCA/MR	dup	3p26.3	176 050	250 850	1	Paternal	B
50	M	MCA/MR	dup	5p14.3	170 578	1 752 211	1	Paternal	B
51	M	MCA/MR	dup	5q13.3	1 020 329	1 421 706	3	Maternal	B
52	M	MCA/MR	dup	7p22.3	568	1 101 943	12	Maternal	B
53	F	MCA/MR	dup	8p23.2	838 610	2 648 539	1	Paternal	B
54	M	MCA/MR	dup	9q33.1	162 612	1 030 807	2	Paternal	B
55	F	MCA/MR	dup	10q22.3	154 664	873 124	1	Maternal	B
56	M	MCA/MR	dup	12q21.31	152 042	4 843 434	3	Paternal	B
57	M	Gillespie syndrome	del	Xp11.23	104 191	115 604	3	Maternal	B

Abbreviations: B, benign; CNV, copy-number variant; F, female; M, male; MCA/MR, multiple congenital anomalies and mental retardation; P, pathogenic.
[a]Two CNVs were detected in case 42.
[b]Two CNVs were detected in case 44.
[c]Nullizygous deletion inherited from his mother probably affected the phenotype.

Another interesting observation in the first screening was that subtelomeric rearrangements frequently occurred even in patients with MCA/MR of uncertain whose karyotype had been diagnosed as normal. This result may be consistent with a property of subtelomeric regions whose rearrangements can be missed in conventional karyotyping,[72] and in fact other techniques involving subtelomeric FISH or MLPA also identified subtelomeric abnormalities in a number of patients with MCA and/or MR in previous reports.[70,73,74] Our result may support the availability of prompt screening of subtelomeric regions for cases with uncertain congenital disorders.

In the second screening we applied WGA-4500 to 349 cases to detect 66 candidate pCNVs in 63 cases (18.1%), and subsequently assessed the pathogenicity of these CNVs. The pCNVs included nine CNVs overlapping identical regions of recently recognized syndromes (cases 1–9; deletion at 1p36.23–p36.22, 1q41–q42.11, 1q43–q44, 2q23.1, 14q12, 15q26-qter and 16p11.2–p12.2, respectively), four CNVs containing disease-associated genes (cases 10–13; *GLI3*, *BMP4*, *YWHAE* and *CASK*, respectively), three pairs of CNVs of recurrent deletions (cases 14, 15: at 6q12–q14.1 and 6q14.1; case 16, 17: at 10p12.1–p11.23 and case 18, 19: at 10q24.31–q25.1 and 10q24.32–q25.1), five CNVs identical to pCNVs in previous studies (cases 20–24), six large and/or gene-rich CNVs (cases 25–30) and six CNVs containing a morbid OMIM gene (cases 31–36). For the remaining cases, we estimated the pathogenicity of the CNVs from a parental analysis (Table 4). We judged the 11 *de novo* CNVs (cases 37–47) and 1 CNV on chromosome Xp11.3 inherited from

the mother (case 48) as probably pathogenic. And nine inherited CNVs (cases 49–57) were probably benign. The clinical significance of CNVs in the other six cases, cases 58–63, remains uncertain (VOUS). As a result we estimated CNVs as pathogenic in 48 cases among 349 cases (13.8%) analyzed in the second screening. None of the pCNVs corresponded to loci of well-established syndromes. This may suggest that our two-stage screening achieved a good balance between rapid screening of known syndromes and investigation of CNV of uncertain pathogenicity.

Table 5 Summary of parental analyses

		Average size (bp)		*The average number of protein-coding genes*
		Min.	*Max.*	
Pathogenic CNVs[a]				
del	23	3 309 267	4 597 689	43
dup	2	1 190 098	1 587 722	61
Total	25	3 139 733	4 356 892	44
Benign CNVs[b]				
del	3	538 481	1 030 504	10
dup	8	334 432	1 740 327	3
Total	11	390 082	1 546 739	5

Abbreviation: CNV, copy-number variant.
[a]Twenty-four *de novo* CNVs and case 48.
[b]Eleven inherited CNVs other than case 48.

Among the cases with parental analyses, the 25 pCNVs had larger sizes and contained more protein-coding genes (average size, 3.1 Mb at minimum to 4.4 Mb at maximum; average number of genes, 44) as compared with the 11 inherited bCNVs that were probably unrelated to phenotypes (average size, 0.39 Mb at minimum to 1.5 Mb at maximum; average number of genes, 5) (Table 5). Although all of the 25 pCNVs except 2 were deletions, about three quarters (8 of 11 cases) of the inherited bCNVs were duplications (Table 5). These findings are consistent with previously reported features of pCNVs and bCNVs.[21,38]

We also compared our current study with recent aCGH studies meeting the following conditions: (1) a microarray targeted to whole genome was applied; (2) patients with MCA and/or MR of uncertain etiology, normal karyotype and the criteria for patients selection were clearly described; (3) pathogenicity of identified CNVs were assessed. On the basis of the above criteria, among studies reported in the past 5 years, we summarized 13 studies (Table 6).[10,14,15,17,54,55,75–81] Diagnostic yield of pCNVs in each study was 6.3–16.4%, and our current diagnostic yield of the second screening was 13.8%. Though cases with subtelomeric aberration detected in the first screening had been excluded, our diagnostic yield was comparable to those of the reported studies. It is not so important to make a simple comparison between diagnostic yields in different studies as they would depend on the conditions of each study, for example, sample size or array resolution,[38,82] however it seems interesting that the higher resolution of a microarray does not ensure an increase in the rate of detection of pCNVs. One recent study showed data that may explain the discrepancy between the resolution of microarray and diagnostic yield.[54,83] The authors analyzed 1001 patients with MCA and/or MR using one

Table 6 Previous studies of analyzing patients with MCA and/or MR using aCGH targeted to whole genome

	Applied array			*Patients*		*Pathogenic CNV*	
Author (year)	*Type*	*Number*[a]	*Distribution*[b]	*Number*	*Type of disorders*	*Number*	*%*
Schoumans *et al.*[75]	BAC	2600	1.0 Mb*	41	MCA and MR	4	9.8
de Vries *et al.*[76]	BAC	32 477	Tiling	100	MCA and/or MR	10	10.0
Rosenberg *et al.*[77]	BAC	3500	1.0 Mb*	81	MCA and MR	13	16.0
Krepischi-Santos *et al.*[78]	BAC	3500	1.0 Mb*	95	MCA and/or MR	15	15.8
Friedman *et al.*[14]	SNP	Affymetrix 100K	23.6 kb**	100	MR	11	11.0
Thuresson *et al.*[79]	BAC		1.0 Mb*	48	MCA and MR	3	6.3
Wagenstaller *et al.*[80]	SNP	Affymetrix 100K	23.6 kb**	67	MR	11	16.4
Fan *et al.*[55]	Oligo	Agilent 44K	24 kb–43 kb**	100[c]	MCA and MR, Autism	15[d]	15.0
Xiang *et al.*[15]	Oligo	Agilent 44K	24 kb–43 kb**	40[e]	MR, DD and autism	3	7.5
Pickering *et al.*[10]	BAC	2600	1 Mb*	354[f]	MCA and/or MR	36[g]	10.2
McMullan *et al.*[17]	SNP	Affymetrix 500K	2.5 kb–5.8 kb**	120	MCA and/or MR	18	15.0
Bruno *et al.*[81]	SNP	Affymetrix 250K	2.5 kb–5.8 kb**	117	MCA and/or MR	18	15.4
Buysse *et al.*[54]	BAC	3431	1 Mb*	298	MCA and/or MR	26	8.7
	Oligo	Agilent 44K	24 kb–43 kb**	703	MCA and/or MR	74	10.5
Our current study	BAC	4523	0.7 Mb	349	MCA and MR	48	13.8
Total				2613		305	11.7

Abbreviations: BAC, bacterial artificial chromosome; CNV, copy-number variant; DD, developmental delay; MCA, multiple congenital anomalies; MR, mental retardation; SNP, single nucleotide polymorphism.
[a]The number of clones or name of array is described.
[b]Each distribution referred to each article (*) or manual of each manufacturer (**).
[c]All cases were analyzed by both a targeted array and a genome-wide array.
[d]In five cases, CNVs were also identified by a targeted array.
[e]Ten cases with an abnormal karyotype were excluded.
[f]Only cases studied with an array throughout the genome are described. Ninety-eight cases were also analyzed by a targeted array.
[g]Seventeen cases with an abnormal karyotype were excluded.