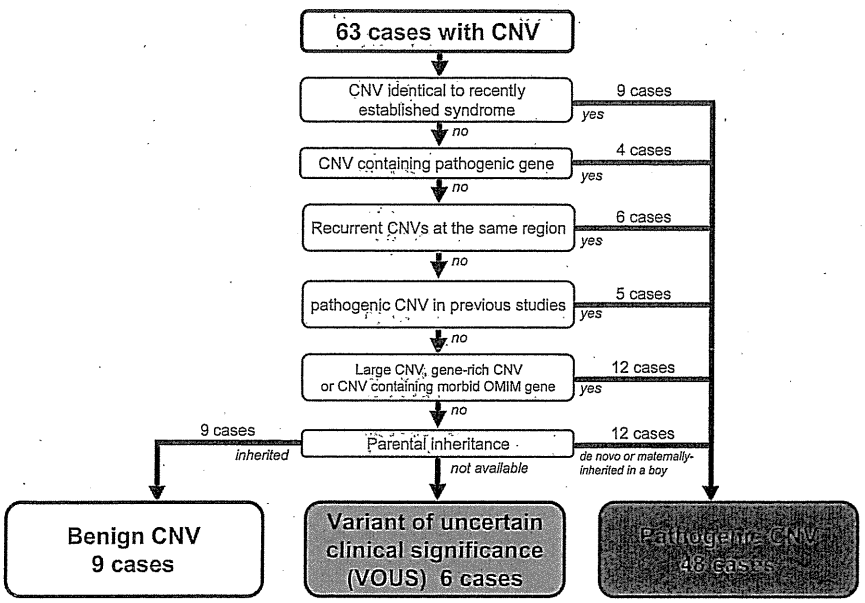

Figure 2 A flowchart of the assessment of CNVs detected in the second screening.

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.

of two types of microarray, BAC array and oligonucleotide array. The BAC array was applied for 298 patients to detect 58 CNVs in 47 patients, and among them 26 CNVs (8.7%) were determined to be causal (pathogenic). Conversely, the oligonucleotide arrays were applied for 703 patients to detect 1538 CNVs in 603 patients, and among them 74 CNVs (10.5%) were determined to be pathogenic. These results may lead to the following idea: a lower-resolution microarray detects a limited number of CNVs likely to be pathogenic, because such CNVs tend to be large, and a higher-resolution microarray detects an increasing number of bCNVs or VOUS.[38] Indeed, in studies using a high-resolution microarray, most of the CNVs detected were smaller than 500 kb but almost all pCNVs were relatively large.[54,81,83] Most of the small CNVs were judged not to be pathogenic, and the percentage of pCNVs stabilized at around 10%. This percentage may suggest a frequency of patients with MCA/MR caused by CNV affecting one or more genes, other than known syndromes and subtelomeric aberrations. The other patients may be affected by another cause undetectable by genomic microarray; for example a point mutation or microdeletion/duplication of a single gene, aberration of microRNA, aberration of methylation states, epigenetic aberration or partial uniparental disomy.

As recently hypothesized secondary insult, which is potentially another CNV, a mutation in a phenotypically related gene or an environmental event influencing the phenotype, may result in clinical manifestation.[84] Especially, in two-hit CNVs, two models have been hypothesized: (1) the additive model of two co-occurring CNVs affecting independent functional modules and (2) the epistatic model of two CNVs affecting the same functional module.[85] It also suggests difficulty in selecting an optimal platform in the clinical screening. Nevertheless, information on both pCNVs and bCNVs detected through studies using several types of microarrays is unambiguously significant because an accumulation of the CNVs will create a map of genotype–phenotype correlation that would determine the clinical significance of each CNV, illuminate gene function or establish a new syndrome.

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ORIGINAL ARTICLE

Breakpoint determination of X;autosome balanced translocations in four patients with premature ovarian failure

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Premature ovarian failure (POF) is a disorder characterized by amenorrhea and elevated serum gonadotropins before 40 years of age. As X chromosomal abnormalities are often recognized in POF patients, defects of X-linked gene may contribute to POF. Four cases of POF with t(X;autosome) were genetically analyzed. All the translocation breakpoints were determined at the nucleotide level. Interestingly, *COL4A6* at Xq22.3 encoding collagen type IV alpha 6 was disrupted by the translocation in one case, but in the remaining three cases, breakpoints did not involve any X-linked genes. According to the breakpoint sequences, two translocations had microhomology of a few nucleotides and the other two showed insertion of 3–8 nucleotides with unknown origin, suggesting that non-homologous end-joining is related to the formation of all the translocations.
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Keywords: *COL4A6*; critical region; non-homologous end-joining; premature ovarian failure; X;autosome translocation

INTRODUCTION

Premature ovarian failure (POF) is a disorder characterized by amenorrhea and elevated serum gonadotropins before 40 years of age. The risk of this disorder or natural menopause before 40 years is approximately 1% of women.[1] Heterogeneous etiology should be involved in POF, such as environmental, autoimmune and genetic factors. X chromosomal abnormalities (partial monosomies and X;autosome-balanced translocations) are often observed in POF patients. These rearrangements cluster at Xq13–q26 called the critical region (for POF).[2,3] The critical region is separated into two: critical region 1 at Xq13–q21 and critical region 2 at Xq23–q26.[2,4] It was suggested that several X-linked loci expressing on both X chromosomes, which were required in a higher dosage for normal ovarian function, were involved in POF.[5] Furthermore, genetic factors for POF may be more complex as X;autosome translocations often disrupt no genes; therefore, other factors, such as position effects on autosomal genes, are proposed.[6] We had an opportunity to analyze four cases of POF each having t(X;autosome). Precise determination of translocation breakpoints in these patients may reveal direct evidence of POF-related genes and mechanisms of the formation of chromosomal translocations. Breakpoint sequences will be presented and discussed in relation to genes and formation process.

MATERIALS AND METHODS

Patients and genomic DNA preparation

A total of four POF patients with t(X;autosome) were recruited to this study. Case 1 had secondary amenorrhea and the other three (cases 2, 3 and 4) presented with primary amenorrhea. Cases 1, 3 and 4 are Japanese and case 2 is Caucasian. Case 2 was reported previously.[7] Chromosome analysis revealed 46,X,t(X;4)(q21.3;p15.2) in case 1, 46,X,t(X;2)(q22;p13) in case 2, 46,X,t(X;4)(q22.1;q12) in case 3 and 46,X,t(X;14)(q24;q32.1) in case 4. All translocations occurred *de novo*. In addition, 11 other POF patients were collected to check candidate gene abnormality. After informed consent was obtained, genomic DNA was prepared from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan). Institutional review board approved the research protocol.

Fluorescence *in situ* hybridization

Metaphase chromosomes were prepared from peripheral blood lymphocytes of POF cases. Bacterial artificial chromosome DNA was labeled with fluorescein isothiocyanate- or Cy3-11-dUTP by Vysis Nick Translation kit (Vysis, Downers

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Grove, IL, USA), and denatured at 70 °C for 10 min. Probe–hybridization mixtures (15 µl) were applied to chromosomes, incubated at 37 °C for 16–72 h, and then washed and mounted in antifade solution (Vector, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole. Photographs were taken on an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Southern blot and inverse PCR

Genomic DNA was digested with restriction enzymes: *Eco*RI and *Hin*dIII for case 1 and her parents, *Sac*I and *Eco*RI for case 2 and a normal female control, *Bam*HI and *Eco*RI for case 3 and her parents and *Nde*I and *Bgl*II for case 4 and her mother. Probes were made by PCR and labeled using DIG synthesis kit (Roche Applied Science, Basel, Switzerland). Hybridization, wash and detection were performed according to the manufacturer's protocol. Inverse PCR was performed using self-ligated DNA after digestion with *Eco*RI (cases 1 and 3), *Sac*I (case 2) and *Bgl*II (case 4). All the breakpoints were determined by sequencing inverse PCR products. Information of primers used is available on request.

Mutation analysis

Genomic DNA was obtained from peripheral blood leukocytes by standard methods and used for mutational screening. Protein coding exons of *COL4A6* (exons 1–45), insulin-like growth factor binding protein 7 (*IGFBP7*) (exons 1–5) and *C14orf159* (exons 4–16) were screened by high-resolution melt analysis using LightCycler 480 system II (Roche Applied Science, Tokyo, Japan), except for exon 1 of *IGFBP7*, which were analyzed by direct sequencing. PCR mixture contained 20 ng genomic DNA, 1× ExTaq buffer, 0.2 mM each dNTPs, 0.3 µM each primer, 0.25 µl SYTO9 (Invitrogen, Carlsbad, CA, USA) and 0.25 U ExTaq HS (Takara, Ohtsu, Japan). PCR was initially denatured at 94 °C for 2 min and cycled 45 times for 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C, and then finalized at 72 °C for 1 min. High-resolution melt analysis was then performed. For exon 1 of *IGFBP7*, PCR mixture contained 20 ng genomic DNA, 1× GC buffer II, 0.4 mM each dNTPs, 1 µM each primers, 2% dimethylsulfoxide and 0.04 U LaTaq HS (Takara), and then PCR was initially denatured at 94 °C for 2 min and cycled 35 times at 94 °C for 20 s, at 60 °C for 20 s, at 72 °C for 1 min, and then finalized at 72 °C for 2 min. If a sample showed an aberrant melting curve pattern, the PCR product was purified using ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced by a standard method using BigDye terminator ver.3 (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Sequences were compared with reference sequences using SeqScape version 2.7 (Applied Biosystems).

X-inactivation assay

Human androgen receptor assay and FRAXA locus methylation assay were performed as described previously,[8,9] with a slight modification. In brief, genomic DNA of patients, their parents and a female control was digested with two methylation-sensitive enzymes, *Hpa*II and *Hha*I. Subsequently, PCR was performed using digested and undigested DNA with human androgen receptor assay primers (FAM-labeled ARf: 5′-TCCAGAATCTGTTCCAGAG CGTGC-3′; ARr: 5′-CTCTACGATGGGCTTGGGGAGAAC-3′)[10] and FRAXA primers (FAM-labeled FRM1f: 5′-AGCCCCGCACTTCCACCACCAGCTCCT CCA-3′; FMR1r: 5′-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′), electrophoresed on ABI PRISM 3100 Genetic analyzer and analyzed with GeneMapper™ Software version 3.5 (Applied Biosystems).

RESULTS

Breakpoint sequences

Using fluorescence *in situ* hybridization analysis of metaphase chromosomes, we could identify Bacterial artificial chromosome clones spanning translocation breakpoints in each patient: RP11-636H11 at Xq22.3 (case 1), RP11-815E21 at Xq22.3 (case 2), RP11-589G9 at 4q12 (case 3) and RP11-904N19 at Xq24 (case 4). Southern blot analysis could identify aberrant bands in all the patients (Figure 1) and subsequent inverse PCR successfully cloned all breakpoints in the four cases. Breakpoint sequences are shown in Figure 2. Junction sequencing of der(X) and der(4) in case 1 revealed a 4192-bp deletion of chromosome X (UCSC genome browser coordinates March 2006 version: chr. X: 107 322 866–107 327 057 bp) and 7082-bp deletion (chr. 4: 11 846 359–11 853 387 bp) of chromosome 4, respectively. In addition, five unknown nucleotides were recognized at the der(X)

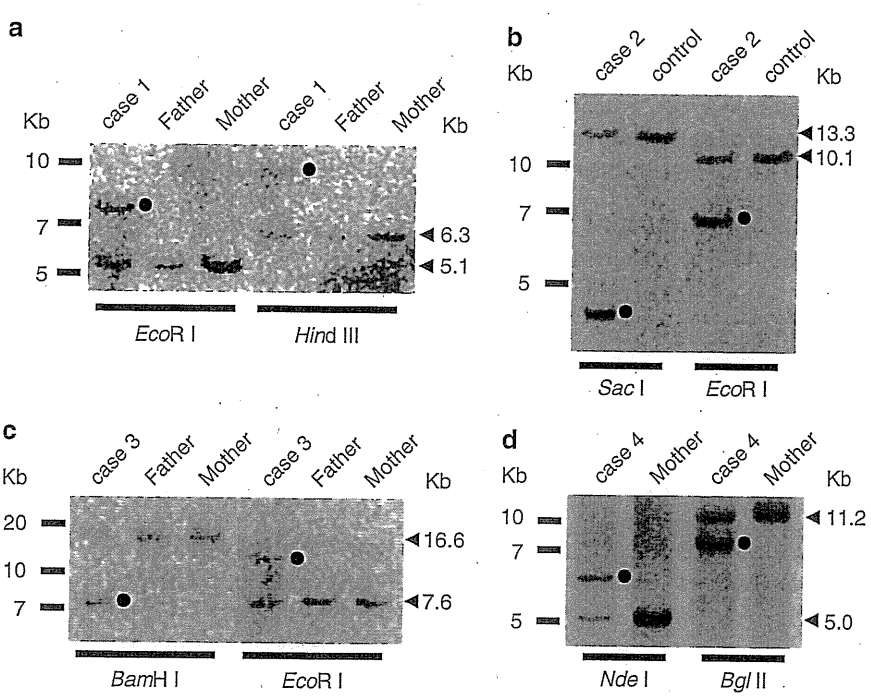

Figure 1 Southern blot analysis of four cases. (a) case 1, (b) case 2, (c) case 3 and (d) case 4. Aberrant bands are indicated with dots.

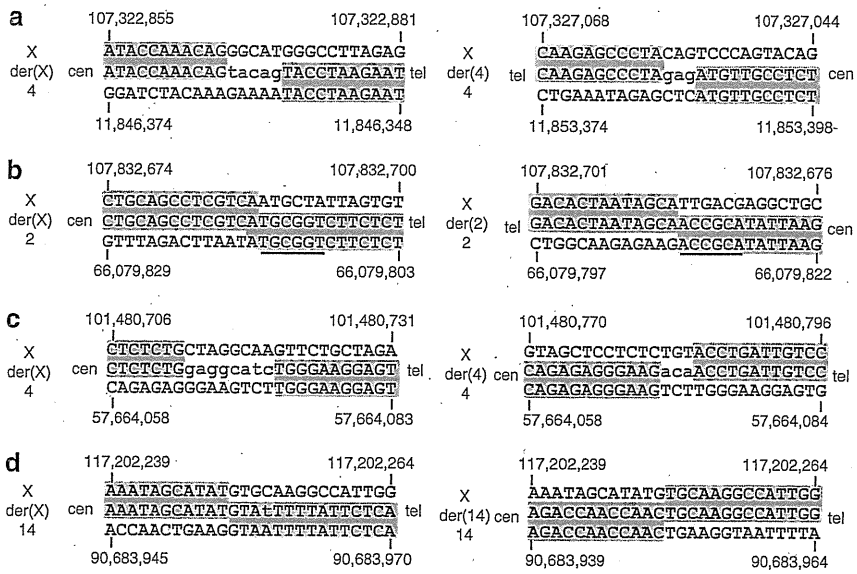

Figure 2 Breakpoint sequences of t(X;autosome) in four cases. (a) case 1, (b) case 2, (c) case 3 and (d) case 4. Top, middle and bottom sequences indicate those of normal X, derivative and normal autosomal chromosomes. Upper and lower cases indicate nucleotides of known and unknown origin, respectively. Matched sequences are with gray shadow. Underline indicates duplicated sequence. Numbers are based on the nucleotide position of the UCSC genome browser coordinates March 2006 version.

junction as well as three unknown nucleotides at der(4) (Figure 2a). Sequences of der(X) and der(2) in case 2 indicated six nucleotides (chr. 2: 66 079 810–66 079 816 bp) were duplicated (Figure 2b). In case 3, 71 nucleotides (chr. X: 101 480 713–101 480 782 bp) were deleted, and unknown eight nucleotides were inserted in der(X), and unknown three nucleotides were also recognized in der(4) (Figure 2c). In case 4, a nucleotide in chromosome X (chr. X: 117 202 250 bp) and five nucleotides in chromosome 14 (chr. 14: 90 683 951–90 683 955 bp) were missing (Figure 2d). The locations of X-chromosome breakpoints are shown in Figure 3. Translocation breakpoints disrupted *COL4A6* at Xq22.3 in case 1 (Figures 3a and b, Table 1), *IGFBP7* at 4q12 in case 3 (Table 1) and *C14orf159* at 14q32.12 in case 4 (Table 1). Other breakpoints did not involve any functional genes. Adjacent genes to breakpoints (less than 100 kb away) are *COL4A5* (5 kb away at Xq22.3) and *IRS4* (30 kb away at Xq22.3) in case 2, *NXF2* (12 or 21 kb away at Xq22.1) in case 3 and *KLHL13* (67 kb away at Xq24) in case 4 (Table 1). *COL4A6*, encoding collagen type IVα6, was the only disrupted X-linked gene in our POF patients.

X-inactivation assay

Human androgen receptor assay in cases 2 and 3 and FRAXA assay in cases 1 and 4 clearly indicated skewed X inactivation in all cases (100% in case 1, 94% in case 2, 98% in case 3 and 100% in case 4) and random patterns in their mothers available for this study (20–80%). Eleven other POF patients also showed random inactivation patterns (30–70%).

Mutation search

Considering accumulation of X-chromosome structural abnormalities in POF, X-chromosomal genes disrupted by rearrangements would be the primary target of this study. As *COL4A6* was completely disrupted in case 1 (Figure 3b), we started analyzing *COL4A6* as a candidate in 11 other POF patients. We found one heterozygous missense change, c.1460G>T (p.Gly487Val) (Figure 3c). This mutation was not recognized in 247 ethnically matched female controls (494 alleles). Web-based SIFT (http://sift.jcvi.org/) and PolyPhen (http://genetics.bwh.harvard.edu/pph/) did not indicate harmful effects of the amino-acid change on protein function: 0.26 by SIFT (predictable functional damage is <0.05) and 'benign' by PolyPhen, but the amino acid was evolutionally conserved (Figure 3d). The Gly487 was located between the (Gly–*X*–*Y*)*n* repeats. Parental origin of the change could not be confirmed as parental samples were unavailable. As *IGFB7* at 4q12 and *C14orf159* at 14q32.12 were also disrupted, both genes were analyzed in the 11 POF patients, but no mutation was found.

DISCUSSION

In this study, we could successfully determine the translocation breakpoints at nucleotide level in all the four cases analyzed. *COL4A6* at Xq22.3 in case 1, *IGFBP7* at 4q12 in case 3 and *C14orf159* at 14q32.12 in case 4 were disrupted. No genes were disrupted in case 2. Importantly, *COL4A6* was the only X-linked gene that was our primary target as a causative gene for POF. One missense change with benign nature outside the functional repeats was found in another POF patient who showed random X inactivation (35%).

In case 1, based on the skewing of X inactivation, der(X) should be active and normal X should be inactive. Thus, *COL4A6* is predicted to be functionally null in case 1 as the active allele is disrupted by the translocation. Collagen type IV is an essential component of basement membrane, consisting of six distinct α-chains (α1–α6) encoded by *COL4A1* to *COL4A6*. These six genes are located in three pairs with head-to-head orientation, *COL4A1*/*COL4A2* on chromosome 13, *COL4A3*/*COL4A4* on chromosome 2 and *COL4A5*/*COL4A6* on chromosome X. The chains interact and assemble with specificity to form three distinct patterns: α1α1α2, α3α4α5 and α5α5α6.[11] The α5- and α6-chains are found in the basement membrane of skin, smooth muscle and kidney.[12] Two transcripts of *COL4A6* are known, isoforms A and B (Figure 3b). The protein structure of collagen type IV contains an amino-terminal collagenous domain (also called 7S domain), a triple-helical region (Gly–*X*–*Y*) and a carboxyl-terminal

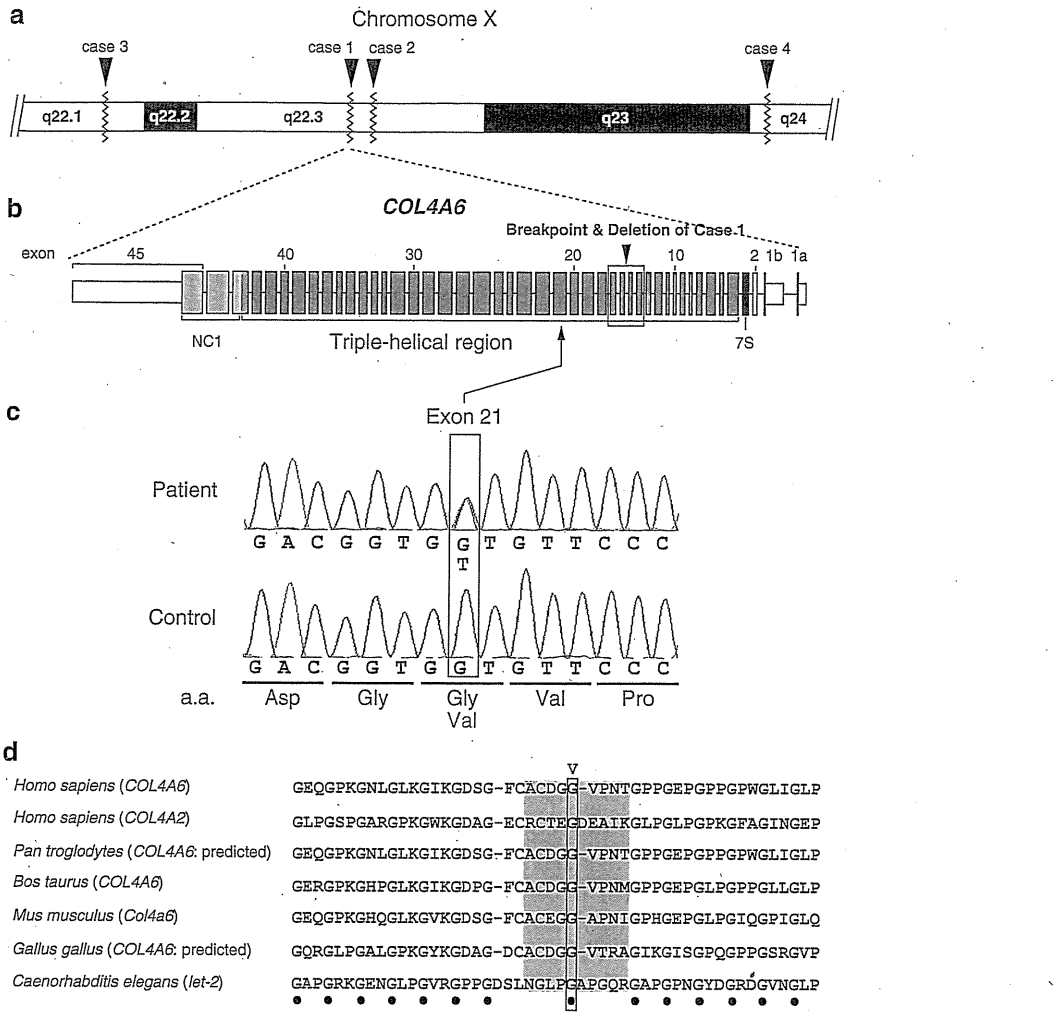

Figure 3 Location of the X-chromosome breakpoints and the *COL4A6* gene. (**a**) Breakpoint locations (zigzag lines) of four cases around Xq22.1–q24. (**b**) Schema of the *COL4A6* gene. Boxes are exons with numbering. White, dark gray, gray and light gray boxes indicate UTRs, 7S domain, triple-helical region and non-collagenous (NC1) domain, respectively. Breakpoint of the translocation with associated deletion is shown above boxes. (**c**) Heterozygous missense mutation, c.1460G>T (p.Gly487Val) at exon 21, is shown in the upper panel and wild-type sequence is shown in the lower panel. a.a.: amino acid. (**d**) Evolutionary conservation of the Gly487. CLUSTALW (http://align.genome.jp/) was used for this analysis. Dots show perfect conservation. Gray box is a space between the Gly–*X*–*Y* repeats.

Table 1 Genes within a 100-kb distance from translocation breakpoints

Case	*Chromosome X*	*Autosome*
1	*COL4A6*	None
	[q22.3]	[4p15.33]
2	*COL4A5* (5 kb)	None
	IRS4 (30 kb)	[2p14]
	[q22.3]	
3	*NXF2*[a] (12 or 21 kb)	*IGFBP7*
	[q22.1]	[4q12]
4	*KLHL13* (67 kb)	*C14orf159*
	[q24]	[14q32.12]

Round and square brackets indicate a distance from a breakpoint and chromosomal location, respectively.
Underlined genes are disrupted by breakpoints.
[a]*NXF2* is mapped to two adjacent segmental duplications.

non-collagenous (NC1) domain (Figure 3b).[13] We found a missense change, c.1460G>T (p.Gly487Val), in exon 21 in another POF patient (Figure 3c). Although this change is not found in 247 Japanese controls, its benign nature is suspected based on the web-based programs, the location outside the functional repeats and random X inactivation leading to the production of normal α6-chain. Parental samples were unfortunately unavailable to test the origin of the nucleotide change.

COL4A6 abnormality is known to be related to Alport syndrome with diffuse leiomyomas (AL-DS). *COL4A6* deletions in AL-DS are limited to exons 1, 1′ and 2 always together with *COL4A5* deletion in diverse extent.[14,15] In this paper, we first describe a POF patient (case 1) with disruption of only *COL4A6* not involving *COL4A5*. The inactivated normal X chromosome as well as the der(X) with disrupted *COL4A6* should lead to functionally null status in the patient. Extracellular matrix proteins (including COL4A6) have been shown to alter Leydig cell steroidogenesis *in vivo*, implying that Leydig cell steroidogenic

activity and matrix environment are interdependent.[16] Therefore, *COL4A6* depletion in ovarian extracellular matrix may alter normal steroidogenesis even in the ovary and have been possibly the cause of POF, especially in case 1. So far, there have been at least eight POF genes registered in OMIM: *FMR1* at Xq27.3 (POF1, OMIM no. 311360), *DIAPH2* at Xq22 (POF2A, OMIM no. 300511), *POF1B* at Xq21 (POF2B, OMIM no. 300604), *FOXL2* at 3q23 (POF3, OMIM no. 608996), *BMP15* at Xp11.2 (POF4, OMIM no. 300510), *NOBOX* at 7q35 (POF5, OMIM no. 611548), *FIGLA* at 2p12 (POF6, OMIM no. 612310) and *NR5A1* at 9q33 (POF7, OMIM no. 312964). Furthermore, *XPNPEP2* at Xq25,[17] *DACH2* at Xq21.2[18] and *CHM* (Xq21.2)[19] have also been described as being disrupted by translocations. *COL4A6* may possibly be an additional X-linked gene related to POF.

Two autosomal genes were disrupted: a gene encoding *IGFBP7* at 4q12 and *C14orf159* on 14q32.12. *IGFBP7* (also known as *IGFBP-rP1* or *MAC25*) is a secreted 31-kDa protein, belonging to the IGFBP superfamily. *IGFBP7* is involved in proliferation, senescence and apoptosis. Recently, it is reported that *IGFBP7* loss has a functional role in thyroid carcinogenesis.[20] *C14orf159* is a hypothetical protein with unknown function. Both disrupted genes were relatively expressed in ovary based on the GeneCards database (http://www.genecards.org/). We could not find any sequence aberrations in either gene among other POF patients. Further analysis might be necessary in relation to POF.

According to the precise breakpoint locations in all the cases reported here, *COL4A5* and *IRS4* (case 2), *NXF2* (case 3) and *KLHL13* (case 4) were localized near to breakpoints (within less than a 100-kb distance). All the adjacent genes except for *KLHL13* are shown to be expressed in human ovary in the GeneCards database. Interestingly, it was suggested that IRS4 protein expression was decreased in theca cells of polycystic ovary syndrome[21] and IGFBP7 protein suppressed estrogen production in granulose cells.[22] Reduced expression of these genes owing to the position effects by translocations could affect to normal ovarian function.

On the basis of the breakpoint sequences, two translocations (in cases 2 and 4) had microhomology (defined as the presence of the same short sequence of bases) of a few nucleotides and the other two (in cases 1 and 3) showed insertion of 3–8 nucleotides of unknown origin, suggesting that non-homologous end-joining is related to the formation of all the translocations in our patients.[23]

In conclusion, we could determine four t(X;autosome) breakpoints at the nucleotide level. We found that only one X-linked gene, *COL4A6*, was disrupted, resulting in functionally null status. All the four translocations are formed by non-homologous end-joining.

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CLINICAL REPORT

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Myelodysplastic Syndrome in a Child With 15q24 Deletion Syndrome

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15q24 deletion syndrome is a recently-described chromosomal disorder, characterized by developmental delay, growth deficiency, distinct facial features, digital abnormalities, loose connective tissue, and genital malformations in males. To date, 19 patients have been reported. We report on a 13-year-old boy with this syndrome manifesting childhood myelodysplastic syndrome (MDS). He had characteristic facial features, hypospadias, and mild developmental delay. He showed neutropenia and thrombocytopenia for several years. At age 13 years, bone marrow examination was performed, which showed a sign suggestive of childhood MDS: mild dysplasia in the myeloid, erythroid, and megakaryocytic cell lineages. Array comparative genomic hybridization (array CGH) revealed a de novo 3.4 Mb 15q24.1q24.3 deletion. Although MDS has not been described in patients with the syndrome, a boy was reported to have acute lymphoblastic leukemia (ALL). The development of MDS and hematological malignancy in the syndrome might be caused by the haploinsufficiency of deleted 15q24 segment either alone or in combination with other genetic abnormalities in hematopoietic cells. Further hematological investigation is recommended to be beneficial if physical and hematological examination results are suggestive of hematopoietic disturbance in patients with the syndrome.

Key words: 15q24 deletion syndrome; thrombocytopenia; neutropenia; myelodysplastic syndrome

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INTRODUCTION

Chromosome 15q24 deletion syndrome [OMIM#613406] is a recently-described disorder characterized by developmental delay, growth deficiency, distinct facial features, digital abnormalities, loose connective tissue, and genital malformations in males [Sharp et al., 2007]. Additional features include diaphragmatic hernia, bowel atresia, and congenital heart defects. The syndrome results from the interstitial deletion of the long arm of chromosome 15, typically detected by array comparative genomic hybridization (array CGH). To date, 19 patients with the syndrome have been reported [Smith et al., 2000; Sharp et al., 2007; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2010; Masurel-Paulet et al., 2009; Van Esch et al., 2009; El-Hattab et al., 2010; McInnes et al., 2010]. The patients shared some major clinical features.

Although El-Hattab et al. [2009] described a boy with the syndrome showing acute lymphoblastic leukemia (ALL), the relationship between hematological abnormalities and 15q24 deletion syndrome has not been reviewed. Here, we report on a boy with the syndrome manifesting myelodysplastic syndrome (MDS). MDS is a clonal disorder characterized by ineffective hematopoiesis, frequently progress to acute myeloid leukemia [Niemeyer et al., 2005]. We speculate on the mechanism of hematological abnormality in the syndrome.

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CLINICAL REPORTS

The patient, now a 13-year-old Japanese boy, was born as the second child of healthy nonconsanguineous parents. His mother had an operation for cervical incompetency during pregnancy. At 39 weeks of gestation, he was born by spontaneous delivery. His birth weight was 2,600 g (5th centile), length 49.5 cm (50th centile), and occipital frontal circumference (OFC) 33 cm (50th centile). Hypospadias and malformed ears were noted at birth.

At age 5 months, he presented with recurrent episodes of fever. Laboratory investigations revealed thrombocytopenia (platelet count, $36 \times 10^3/\mu l$). Bone marrow examination showed normal cellular elements with increasing megakaryocytes. The serum level of platelet associated immunoglobulin G was elevated at 160.0 ng/10^7 cells (normal range, 5–25 ng/10^7 cells). His condition was diagnosed as idiopathic thrombocytopenic purpura. The administration of corticosteroid and immunoglobulin infusion improved thrombocytopenia temporarily. However, thrombocytopenia occurred again at age 10 months and persisted up to the present time with occasionally subcutaneous hemorrhage (platelet count between 70 and $100 \times 10^3/\mu l$). He had recurrent respiratory infections with frequent admission in his childhood and school age. At age 10 years, he was first noted as having moderate neutropenia (absolute neutrophil count, $0.84 \times 10^3/\mu l$).

Developmental retardation was evident at age 5 months. He sat unsupported at 12 months, walked at 25 months, and spoke his first word at 24 months. A G-banding chromosomal analysis showed a normal karyotype. Metabolic investigations, cardiac ultrasonography, cranial magnetic resonance imaging, and electroencephalography all obtained normal findings. An ophthalmologic investigation showed mild myopia. An otological examination showed severe bilateral mixed hearing impairment. The Wechsler Intelligence Scale for Children at age 7 years showed mild intellectual disability.

When seen by us at age 13 years, his height was 149.5 cm (10th–25th centile), weight 37.6 kg (10th–25th centile), and OFC 55 cm (50th–75th centile). He had a long face, broad medial eyebrows, epicanthal folds, downslanting palpebral fissures, a flat nasal bridge, a high palate, a smooth philtrum, a full lower lip, cupped ears, short fifth fingers, hypospadias, mild digital joint contractures, and mild scoliosis (Fig. 1). He also showed severe

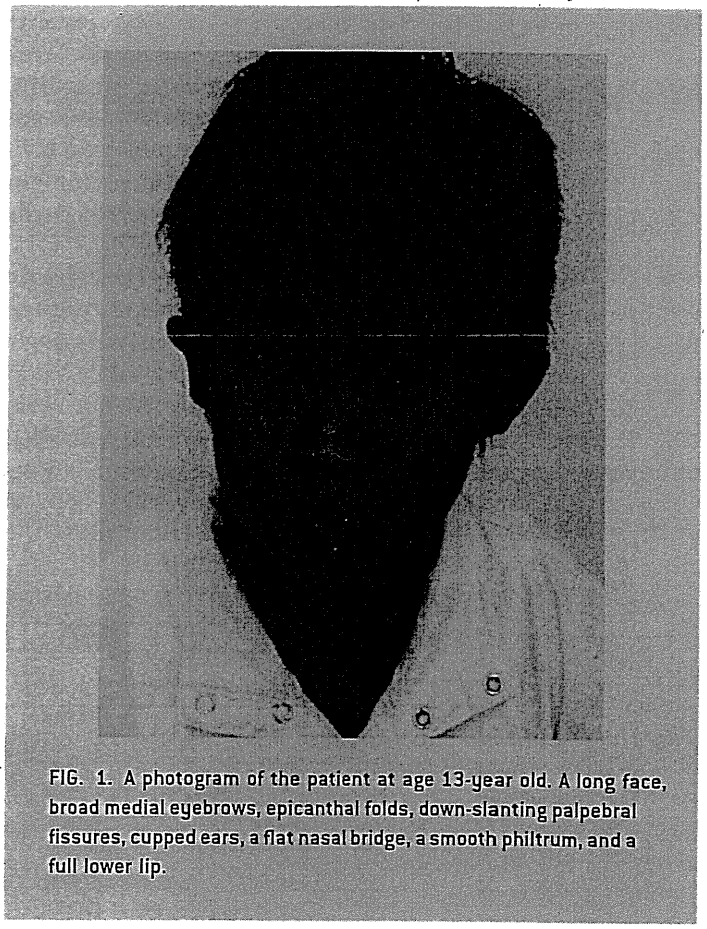

FIG. 1. A photogram of the patient at age 13-year old. A long face, broad medial eyebrows, epicanthal folds, down-slanting palpebral fissures, cupped ears, a flat nasal bridge, a smooth philtrum, and a full lower lip.

obstructive sleep apnea attributable to tonsillar hypertrophy. Hepatosplenomegaly was not observed. Though the hemoglobin level was normal at 13.3 g/dl, total white blood cell count was decreased at $1.9 \times 10^3/\mu l$, absolute neutrophil count was decreased at $0.43 \times 10^3/\mu l$, and platelet count was decreased at $68 \times 10^3/\mu l$. Serum levels of coagulation factors were all normal range. The serum immunoglobulin levels were normal. The results of the autoimmune screening were positive for anti-cardiolipin antibody (17 U/ml; normal range <10 U/ml) and negative for anti-double-stranded DNA antibody, antinuclear antibodies, anti-smith

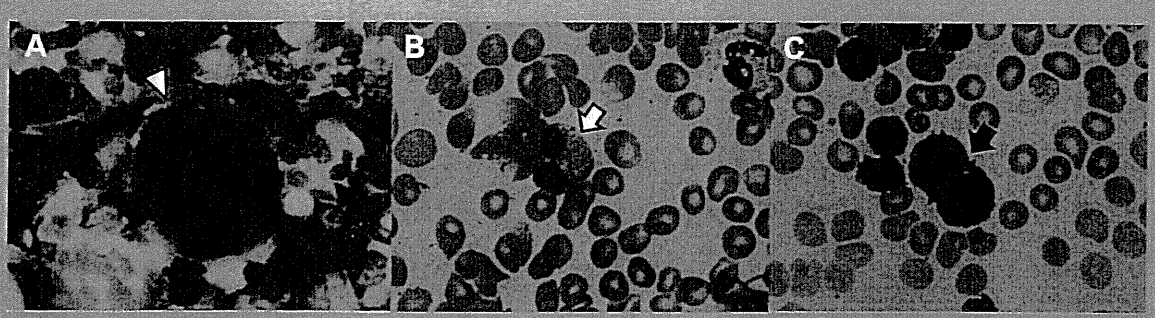

FIG. 2. Bone marrow findings of the present patient. A: Small megakaryocyte is noticeable (white arrow head). B: Pseudo-Pelger abnormality in neutrophil is shown (white arrow). C: Bilobed nuclei in erythroblast is noted (black arrow).

antibody, rheumatoid factor, antiplatelet antibody, platelet-associated immunoglobulin G, and antineutrophil antibody. Serological and polymerase chain reaction testing for parvovirus B19, cytomegalovirus, and Ebstein–Barr virus did not show an evidence of active infection. Chromosomal breakage with mitomycin C in peripheral blood lymphocytes was not increased. Monosomy 7 in bone marrow cells and telomere shortening in peripheral blood cells were not observed by cytogenetic analysis.

A bone marrow examination showed mild trilineage dysplasia with normal cellularity and increased number of megakaryocytes. Blast cell count was three percent. Megakaryocytes with single nuclei and noticeable small size, neutrophils with poor granulation and pseudo-Pelger abnormalities, and erythroblasts with binuclearity and mild hypoplasia were noted (Fig. 2A–C). According to the criteria by the fourth edition of the World Health Organization classification of hematopoietic and lymphoid neoplasms [Baumann et al., 2008], his condition was classified as childhood MDS.

MOLECULAR CYTOGENETIC INVESTIGATIONS

The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Shinshu University School of Medicine, and informed consent was obtained from the parents of the patient.

DNA was extracted using the Gentra Puregene Blood Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Array CGH was performed using the CGX-3 cytogenetics arrays (Roche NimbleGen, Inc., Madison, WI). This platform included 134,829 oligonucleotide probes covering the whole genome at an average resolution of 35 kb as well as clinically significant regions at 10 kb. The procedures for DNA labeling, and hybridization

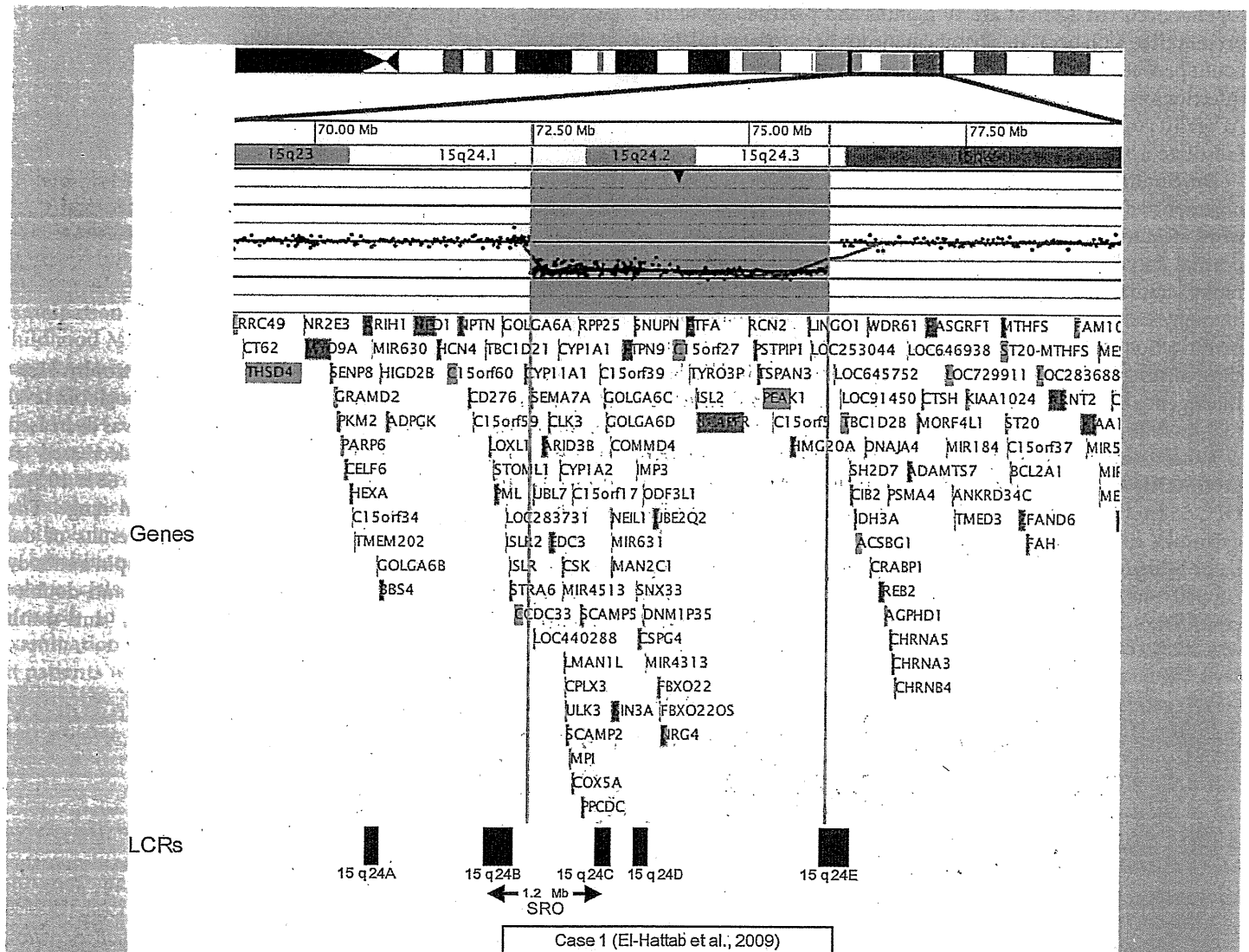

FIG. 3. Array CGH (Roche NimbleGen CGX-3 cytogenetics array) identifying a 3.4-Mb deletion at 15q24.1q24.3. The white box represent the deleted region in Case 1 (72.252–75.937 Mb) with acute lymphoblastic leukemia [El-Hattab et al., 2009]. The smallest region of overlap (SRO) by published patients and the five LCR regions represented by black boxes are shown.

were performed according to the manufacturer's instructions. The slides were scanned into image files using a NimbleGen MS 200 scanner. The array data analysis was performed using Genoglyphix Software (Signature Genomics Laboratories, Spokane, WA). Mapping data were analyzed on the Genoglyphix Genome Browser. All genomic locations correspond to NCBI build 36 (hg18). The array CGH analysis of the patient showed a deletion at chromosome region 15q24.1–15q24.3. The minimum size of the deletion was estimated as 3.44 Mb from the probe at 15q24.1 (chr15: 72,485,279 bp) to the probe at 15q24.3 (chr15: 75,921,984 bp) (Fig. 3).

To confirm array CGH findings, fluorescence in situ hybridization (FISH) analysis was performed on peripheral blood lymphocytes of the patient. BAC clones RP11-195A1 and RP11-91J9 were deleted and permitted to confirm the interstitial deletion on chromosome 15q24.1–q24.3 (data not shown). G-banding chromosomes of the parents were normal. The FISH analysis using two deleted BAC clones detected both two signals on the parental chromosomes. Thus, the aberration observed in present patient occurred de novo.

TABLE I. Clinical Features in the Present Patient Compared to 19 Published Patients With 15q24 Deletion Syndrome

	Present patient	Nineteen previously reported cases
Deletion length	3.44 Mb	1.7–6.1 Mb
	72.48–75.92	67.8–76.08
Inheritance	De novo	17 de novo, 2 unknown
Gender	Male	17 male, 2 female
Age	13y	5mo–33y
Growth retardation	–	5/19
Intellectual disability/ developmental delay	+	19/19
Facial abnormalities		
High forehead/anterior hair line	–	12/19
Long/narrow face	+	5/19
Broad medial eyebrows	+	7/19
Epicanthus folds	+	8/19
Hypertelorism	–	8/19
Downslanting palpebral fissures	+	8/19
Smooth/long philtrum	+	10/19
Full lower lip	+	7/19
Eye abnormalities		
Strabismus	–	7/19
Dysopia	+	3/19
Ear abnormalities		
Hearing impairment	+	4/19
Malformed ear	+	12/19
Brain malformation	–	9/19
Cardiac abnormalities	–	5/19
Urogenital abnormalities	+	11/19
Skeletal malformation		
Joint laxity	–	8/19
scoliosis	+	6/19
Digital abnormalities	+	14/19
Diaphragmatic hernia	–	3/19
Inguinal hernia	–	4/19
Gastrointestinal abnormalities	–	3/19
Recurrent infection	+	7/19
Hematopoietic disorder	+	1/19
Autism spectrum	–	3/19

mo, month; y, year.

DISCUSSION

We report on a 13-year-old boy with characteristic facial features, hypospadias, mild developmental delay manifesting MDS. Array CGH demonstrated 15q24 deletion. This syndrome was recently delineated by Sharp et al. [2007]. Several patients had been described as having deletions encompassing the 15q24 segment, examined by standard chromosomal analysis and FISH studies [Cushman et al., 2005]. They showed many overlapping clinical features with the 15q24 deletion syndrome. However, their breakpoints had not been scrutinized. Most patients with 15q24 deletion syndrome are reported to have recurrent breakpoints, which are supposed to be mediated by nonallelic homologous recombination (NAHR) between the five low-copy repeats (LCRs) region. The deletions ranged from 1.7 to 6.1 Mb, with the smallest region of overlap (SRO) spanning approximately 1.2 Mb (chr15: 72.1–73.3 Mb) [El-Hattab et al., 2010]. Present patient had a deleted region spanning approximately 3.4 Mb, which comprises 50 genes and include the previously delineated SRO (Fig. 3).

To date, 20 patients with 15q24 deletion syndrome, including present patient, have been reported. These patients shared major clinical manifestations, including developmental delay, growth deficiency, characteristic facial dysmorphism, digital abnormalities, loose connective tissue, and genital malformations in males. The patient showed most of the major features reported in previous patients, but did not show growth deficiency, loose connective tissue, or brain anomaly (Table I). The patient manifested MDS, which has never been described in patients with the syndrome. ALL has been described in a boy reported by El-Hattab et al. [2009], whose breakpoints were similar to those of present patient (Fig. 3).

Childhood MDS is a relatively rare and complex disease. It is difficult to diagnose childhood MDS when the blast count is not elevated and clonality cannot be established. Hence, children with myelodysplasia or suspected MDS must be extensively worked up for secondary causes of dyspoiesis including nutritional deficiency, medications, toxins, metabolic diseases, infections, autoimmune diseases, growth factor therapy, and congenital disorders of hematopoiesis. Among them, congenital disorders of hematopoiesis including Fanconi anemia, dyskeratosis congenita, Diamond–Blackfan syndrome, Down syndrome, and mitochondria cytopathy is responsible for 29–44% of pediatric patients in whom MDS develops [McKenna, 2004; Yin et al., 2010]. The examinations on the present patient had no findings to suggest these secondary causes. The bone marrow examination of the patient showed mild trilineage dysplasia. His condition was classified as childhood MDS [Baumann et al., 2008].

MDS progress rapidly to leukemia or slowly over many years. Patients have a deteriorating course with 30% evolving into acute

leukemia usually of myeloid phenotype. Evolution into ALL from MDS is rare and seen in <1% adult cases and extremely rare in pediatric population. However, 26 patients with MDS progress to ALL were reported [Gupta and Bhatia, 2010]. El-Hattab et al. [2009] reported a boy with 15q24 deletion and ALL. They hypothesized some tumor-associated genes which located in 15q24 region, *C-Src* tyrosine kinase (*CSK*) and *SIN3A* may lead to increased risk of developing neoplasm. *UBL7/BMSC-UbP* located at 15q24.1 isolated from the bone marrow stromal cell cDNA library encodes a bone marrow stromal cell-derived ubiquitin-like protein. *UBL7* was suggested to a play role in the regulation of bone marrow stromal cell function or cell differentiation through an evocator and cell specific pattern [Liu et al., 2003]. The development of MDS and hematological malignancy in the syndrome might be caused by the haploinsufficiency of deleted 15q24 segment either alone or in combination with other genetic abnormalities in hematopoietic cells.

In conclusion, we report a patient with MDS and 15q24 deletion syndrome. This syndrome might be prone to have hematological malignancy. A careful hematological follow-up of the present patient is required. Further hematological investigation is recommended to be beneficial if physical and hematological examination results are suggestive of hematopoietic disturbance in patients with 15q24 deletion syndrome.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Neurodevelopmental Features in 2q23.1 Microdeletion Syndrome: Report of a New Patient With Intractable Seizures and Review of Literature

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2q23.1 microdeletion syndrome is a recently characterized chromosomal aberration disorder uncovered through array comparative genomic hybridization (array CGH). Although the cardinal feature is intellectual disability (ID), neurodevelopmental features of the syndrome have not been systematically reviewed. We present a 5-year-old boy with severe psychomotor developmental delay/ID, progressive microcephaly with brain atrophy, growth retardation, and several external anomalies. He manifested intractable epilepsy, effectively treated with combined antiepileptic drug therapy including topiramate. Array CGH demonstrated a de novo interstitial deletion of approximately 1 Mb at 2q23.1–q23.2, involving four genes including *MBD5*. Nineteen patients have been reported to have the syndrome, including present patient. All patients whose data were available had ID, 17 patients (89%) had seizures, and microcephaly was evident in 9 of 18 patients (50%). Deletion sizes ranged from 200 kb to 5.5 Mb, comprising 1–15 genes. *MBD5*, the only gene deleted in all patients, is considered to be responsible for ID and epilepsy. Furthermore, the deletion junction was sequenced for the first time in a patient with the syndrome; and homology of three nucleotides, identified at the distal and proximal breakpoints, suggested that the deletion might have been mediated by recently-delineated genomic rearrangement mechanism Fork Stalling and Template Switching (FoSTeS)/microhomology-mediated break-induced replication (MMBIR).

Key words: 2q23.1 microdeletion syndrome; array CGH; neurological features; epilepsy; *MBD5*; FoSTeS/MMBIR

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INTRODUCTION

The 2q23.1 microdeletion syndrome is a recently characterized chromosomal aberration disorder uncovered by array comparative genomic hybridization (array CGH). To date, only 18 patients have been reported to have the syndrome (Table I) [Vissers et al., 2003; Koolen et al., 2004; de Vries et al., 2005; De Gregori et al., 2007; Wagenstaller et al., 2007; Jaillard et al., 2009; Williams et al., 2010; van Bon et al., 2010; Chung et al., 2011]. The cardinal feature is intellectual disability (ID) with pronounced speech delay. Additional features include coarse face, short stature, microcephaly, seizures, and behavioral abnormalities such as stereotypic

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TABLE I. Neurological and Molecular Findings of Our Patient and 18 Previously Reported Patients With Microdeletion Encompassing the 2q23.1 Region

			Deleted region						Seizures					
Patient [reference]	Sex	Array	Start	End	Size (Mb)	Deleted genes	OFC (centile)	MR	Severity	Type	Onset	AEDs	EEG	MRI
Our patient	M	Agilent Oligo 1M	148.8	149.8	0.992	*MBD5, EPC2, KIF5C, LYPD6B*	<3rd (−3.6 SD)	Severe	Severe	CPS	10M	CBZ, ZNS, CLB, TPM	$F^{\#1}$	Myelination delay, brain atrophy in $F^{\#1}$ and $T^{\#2}$
1 [Wagenstaller et al., 2007, Patient 27737]	M	Affimetrix SNP 100K	149.0	149.2	0.2	*MBD5*	ND	Severe	Drug resistant	ND	1Y4M	ND	ND	ND
2 [Jaillard et al., 2009, Subject 1]	M	Agilent Oligo 44K	148.8–149.1	149.3–149.4	0.3	*MBD5, EPC2*	<3rd (−3 SD)	Severe	ND	ND	3M	ND	Non-specific	Small cerebellar vermis
3 [van Bon et al., 2010, Patient 8a]	M	Agilent Oligo 244K	148.5	148.9	0.4	*ORC4L, MBD5*	25th	Moderate	—	—	—	—	ND	Normal
4 [van Bon et al., 2010, Patient 8b]	F	Agilent Oligo 244K	148.5	148.9	0.4	*ORC4L, MBD5*	25th	Moderate	ND	ND	10Y	ND	ND	Ventricular asymmetry
5 [van Bon et al., 2010, Patient 7]	M	Agilent Oligo 44K	148.7	149.2	0.5	*MBD5, EPC2*	<3rd	ND	Severe epileptic encephalopathy	GTCS, GTS, AS, Ab	3Y	Multiple	Multiregion	Normal
6 [Williams et al., 2010, Case 1]	F	Agilent Oligo 244K	148.4	149.4	~0.93	*ORC4L, MBD5, EPC2, KIF5C*	<3rd	ND	Well controlled	CPS	8Y	OXC	Mild diffuse encephalopathy' changes	Normal
7 [van Bon et al., 2010, Patient 10]	M	Agilent Oligo 244K	148.1	149.2	1.1	*ACVR2A, ORC4L, MBD5, EPC2*	<3rd	Severe	ND	PS, Secondary GS	Newborn	ND	$F^{\#1}$ and $C^{\#3}$	Focal cortical abnormalities in right $T^{\#2}$
8 [van Bon et al., 2010, Patient 9]	F	Affymetrix SNP 250K	148.8	150	1.2	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6*	10th	Severe	Drug resistant	ND	10M	ND	Right $T^{\#2}$ and $O^{\#5}$	Wide frontal ventricles and myelination delay
9 [Koolen et al., 2004]	F	BAC 3.6K	145.4–146.7	148.7–151.1	2.0	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B*	<3rd (−2 SD)	Severe	ND	GS	12Y	ND	ND	Cortical atrophy
10 [van Bon et al., 2010, Patient 5]	F	Agilent Oligo 244K	148.4	151.1	2.7	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	50th	Severe	Drug resistant, and died after several seizures	ND	9M	ND	ND	ND
11 [van Bon et al., 2010, Patient 2]	F	Affymetrix SNP 250K	148.7	151.5	2.8	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	10th	ND	ND	ND	3Y10M	ND	ND	Normal

12 [van Bon et al., 2010, Patient 3]	F	Agilent Oligo 244K	148.1	151	2.9	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	50th	Moderate	—	—	—	—	ND	Normal
13 [van Bon et al., 2010, Patient 4]	M	HumanCNV370 CNV-SNP 370K	147.2	150.1	2.9	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6*	16th	Severe	ND	AS	1Y5M	ND	ND	Thinnng of PCC[#4]
14 [Jaillard et al., 2009, Subject 2]	M	IntegraChips BAC 3K	145.3–146.9	149.3–150.7	2.4–5.4	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	Median	Severe	ND	ND	3Y	VPA	Normal	Hypoplasia of F[#1]
15 [Williams et al., 2010, Case 2]	F	Agilent Oligo 244K	146.8	150.3	3.51	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	15th	ND	Well controlled	GTCS	8M	VPA	Light F[#1]	Normal
16 [Chung et al., 2011]	F	Agilent Oligo 105K/244K	148.9	152.9	3.986	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3, RBM43, NMI, TNFAIP6, RIF1, NEB, ARL5A, CACNB4, STAM2*	<3rd	Moderate, regression at age 6 years	Well controlled	ND	3Y	ND	ND	Normal
17 [van Bon et al., 2010, Patient 6]	M	Cytochip v 3.01 BAC >5K	146.7	151.8	5.2	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	<3rd	ND	ND	Ab, Fs	2Y	ND	Normal	Normal
18 [van Bon et al., 2010, Patient 1]	F	Affymetrix SNP 500K	146.6	152.2	5.5	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3, RBM43, NMI, TNFAIP6, RIF1, NEB*	2nd	ND	ND	ND	ND	ND	MD	White matter abnormalities

MR, mental retardation; AEDs, antiepileptic drugs; ND, not described; CPS, complex partial seizure; AS, atonic seizure; Ab, absence seizure; Fs, febrile seizure; GTCS, generalized tonic-clonic seizure; GTS, generalized seizure; Secondary GS, secondary generalized seizure; PS, partial seizure; CBZ, carbamazepine; ZNS, zonisamide; CLB, clobazam; TPM, topiramate; VPA, valproate; OXC, oxcarbazepine.
F[#1], frontal region; T[#2], temporal region; C[#3], central region; PCC[#4], posterior corpus callosum; O[#5], occipital region.

repetitive behavior, disturbed sleep pattern, and broad-based gait [van Bon et al., 2010].

There have been no reports reviewing neurodevelopmenal features in 2q23.1 microdeletion syndrome. We here present the detailed clinical features and course of a boy with the syndrome who had severe psychomotor developmental delay and ID, progressive microcephaly, and intractable epilepsy that was improved by multi-drug therapy including topiramate (TPM). High-resolution array CGH demonstrated a 992-kb deletion at 2q23.1–q23.2 involving four genes including *MBD5*, and the breakpoint-junction sequencing revealed microhomology of three nucleotides at the distal and proximal breakpoints, suggesting that the deletion might have been mediated by recently delineated genomic rearrangement mechanism Fork Stalling and Template Switching (FoSTeS)/microhomology-mediated break-induced replication (MMBIR).

CLINICAL REPORT

The patient is the third child, with two healthy brothers, of a healthy non-consanguineous 33-year-old mother and 34-year-old father. He was born at 38 weeks of gestation by spontaneous vaginal delivery. His birth weight was 2,960 g (−0.1 SD), length 48.2 cm (−0.2 SD), and OFC 31.5 cm (−1.0 SD). He has suffered from bronchial asthma since infancy. At the age of 10 months, he was referred to our hospital for afebrile clonic seizures involving alternating sides with impaired consciousness. The patient's seizures tended to occur episodically and in clusters. Although he had one episode of status epilepticus for 60 min, most of his seizures were not prolonged. On presentation, his weight, height, and OFC were 9.1 kg (± 0 SD), 71 cm (−0.7 SD), and 42.5 cm (−2.0 SD, Fig. 1a), respectively. He showed psychomotor delay, with a developmental quotient (DQ) of 57 on the Kinder Infant Development Scale (KIDS) [Cheng et al., 2010]. His craniofacial features included brachycephaly, strabismus, a short nose with anteverted nostrils, a short philtrum, macroglossia, a high palate, a bifid uvula, and a submucous cleft palate. Additionally, he had short and curved 5th fingers, a single transverse crease on the right palm, and bilateral undescended testes. Blood levels of lactate and pyruvate and serum levels of thyroid hormones were within normal ranges. Amino acid and organic acid disorders were excluded. A cardiac ultrasonography showed mild supravalvular pulmonary artery stenosis. An interictal EEG showed sporadic spikes in the frontal region during sleep with normal background activity (Fig. 1b). A brain MRI showed no obvious abnormalities and a normal myelination pattern.

The patient's clinical course of epilepsy is shown in Fig. 1c. Carbamazepine (CBZ) was started at the age of 10 months. Seizure frequency and duration decreased, but he began exhibiting stereotypical characteristics of frontal lobe epilepsy (FLE): motion and speech arrested with mild rigidity of the upper limbs and incomplete loss of consciousness for 40–60 sec. Zonisamide (ZNS) resulted in a slight reduction of seizure frequency and clobazam (CLB) controlled his condition for several months, although the seizures restarted with cluster attacks. At the age of 4 years, TPM altered from ZNS reduced his seizure frequency and intensity, disappeared cluster attacks, and shortened (<30 sec) the durations. An interictal EEG showed many paroxysmal discharges with slow wave in the frontal region (Fig. 1d). A brain MRI at the age of 5 years showed delayed myelination and mild brain atrophy (Fig. 1e).

Microcephaly was evident after age 1 (Fig. 1a). The patient could roll over and sit without support at the age of 9 months, walk independently at 21 months, and jump at 4 years. When last examined at the age of 5 years, he weighed 15.2 kg (−1.0 SD) and had a height of 98.3 cm (−2.0 SD), and OFC of 45.3 cm (−3.4 SD). He could produce several words but no sentences. Although he had no regressive psychomotor changes, his DQ as evaluated by KIDS had dropped to 24 from 46 at age 2.

CYTOGENETIC AND MOLECULAR ANALYSIS

G-banded chromosomal analysis (550 bands level) using the patient's peripheral blood leukocytes showed a normal karyotype (46,XY). Array CGH analysis with Agilent 1M array (Agilent Technologies, Inc., Santa Clara, CA) demonstrated a 992-kb heterozygous deletion at 2q23.1–q23.2 (USCS hg18, Mar. 2006, chromosome 2: 148,830,937–149,823,345 bp) (Fig. 2a). The deletion was confirmed with FISH using probes originated from four BACs (RP11-295N18 at 2q22.3, RP11-375H16 at 2q23.1, RP11-1005D13 at 2q23.2, and RP11-714O10 at 2q23.3): RP11-375H16 and RP11-1005D13 were deleted, whereas RP11-295N18 and RP11-714O10 were present (Fig. 2b). Subsequently, the deletion junction was amplified by a long PCR (Fig. 2c; sequences of the primer set are available on request) and its product was directly sequenced (Fig. 2d). Three nucleotides (CTG) were shared by sequences at the proximal breakpoint and the distal breakpoint in normal chromosome 2 (Fig. 2d). FISH analysis using the four BAC probes (data not shown) and the junction PCR on parental samples (Fig. 2c) showed that the deletion had occurred de novo. Therefore, the karyotype was concluded as 46,XY.arr 2q23.1q23.2-(148,830,937–149,823,345) × 1 dn, and he was diagnosed with 2q23.1 microdeletion syndrome involving four genes: *MBD5*, enhancer of polycomb, drosophila, homolog of 2 (*EPC2*), kinesin family member 5C (*KIF5C*), and LY6/PLAUR domain containing 6B (*LYPD6B*).

DISCUSSION

The patient we have described had severe psychomotor developmental delay and ID with progressive microcephaly and intractable seizures improved by multi-drug therapy including TPM. Roughly 1 Mb region at 2q23.1–q23.2, which involved four genes including *MBD5*, was found to be deleted and both the proximal and the distal breakpoints were demonstrated to share microhomology of three nucleotides.

The neurological and molecular cytogenetic findings of 19 patients with the syndrome, including the present patient, are shown in Table I. Microcephaly (OFC < 3rd centile) was evident in 9 of 18 patients (50%), whereas younger patients with OFC > 3rd centile might exhibit microcephaly thereafter as demonstrated in the present patient. Moderate to severe ID was noted in all patients whose data were available. Seventeen patients (89%) had seizures: generalized seizures in five patients, partial seizures in three, and seizures of unspecified nature in nine. Median age of seizure onset