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ORIGINAL ARTICLE

Copy-number variations on the X chromosome in Japanese patients with mental retardation detected by array-based comparative genomic hybridization analysis

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X-linked mental retardation (XLMR) is a common, clinically complex and genetically heterogeneous disease arising from many mutations along the X chromosome. Although research during the past decade has identified >90 XLMR genes, many more remain uncharacterized. In this study, copy-number variations (CNVs) were screened in individuals with MR from 144 families by array-based comparative genomic hybridization (aCGH) using a bacterial artificial chromosome-based X-tiling array. Candidate pathogenic CNVs (pCNVs) were detected in 10 families (6.9%). Five of the families had pCNVs involving known XLMR genes, duplication of Xq28 containing *MECP2* in three families, duplication of Xp11.22-p11.23 containing *FTSJ1* and *PQBP1* in one family, and deletion of Xp11.22 bearing *SHROOM4* in one family. New candidate pCNVs were detected in five families as follows: identical complex pCNVs involved in dup(X)(p22.2) and dup(X)(p21.3) containing part of *REPS2*, *NHS* and *IL1RAPL1* in two unrelated families, duplication of Xp22.2 including part of *FRMPD4*, duplication of Xq21.1 including *HDX* and deletion of Xq24 noncoding region in one family, respectively. Both parents and only mother samples were available in six and three families, respectively, and pCNVs were inherited from each of their mothers in those families other than a family of the proband with deletion of *SHROOM4*. This study should help to identify the novel XLMR genes and mechanisms leading to MR and reveal the clinical conditions and genomic background of XLMR.
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INTRODUCTION

Mental retardation (MR) is characterized by nonprogressive cognitive impairment and affects 1–3% of the general population. The predominance of males in the MR population has been attributed to genes located on the X chromosome. In fact, individual X-linked genes were recently estimated to contribute to 10–12% of all MR cases in males.[1] X-linked MR (XLMR) conditions have been divided into syndromic (MRXS representing approximately one-third of XLMR) and nonsyndromic (MRX representing approximately two-third of XLMR).[2] As MRX have no obvious and consistent phenotypes other than MR, XLMR conditions are clinically diverse and genetically heterogeneous disorders. In excess of 215 XLMR conditions have been recorded (http://xlmr.interfree.it/home.htm and http://www.ggc.org/xlmr.htm) and 90 XLMR genes have been identified.[3,4] Genes for 87 conditions have been mapped by linkage analysis and/or cytogenetic breakpoints, but for 38 conditions, genes have been neither identified nor mapped to candidate loci. In addition, more than 300 X-linked protein-coding genes are expressed in brain tissue, suggesting that many XLMR genes remain to be unidentified.[5]

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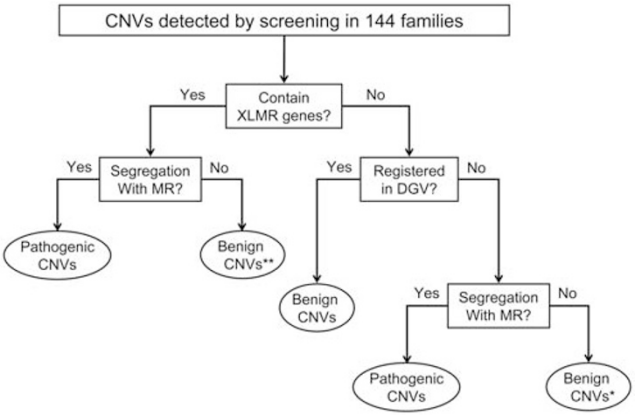

Figure 1 The flowchart of the screening of MR-associated pathogenic CNV. DGV means database of genomic variants. Asterisks indicate types of benign CNVs corresponding to asterisks in Supplementary Table S1.

Array-based comparative genomic hybridization (aCGH) has revealed copy-number variations (CNVs) to be the cause of MR.[6–8] Although Tarpey *et al*.[4] screened for mutations in the coding regions of 718 genes on the X chromosome in probands from 208 families by means of resequencing, only three XLMR-associated genes have been identified, suggesting structural variations other than point mutations, including CNVs or variants in regulatory regions, to contribute to unidentified XLMR conditions.

In this study, we examined CNVs in individuals with MR from 144 families with at least one affected male by aCGH using an in-house bacterial artificial chromosome (BAC)-based X-tiling array (MCG X-tiling array).[9] We detected 10 candidate pathogenic CNVs (pCNVs) according to a flowchart of our procedure (Figure 1), suggesting that pCNVs on the X chromosome could be found at a constant rate by the high-density aCGH in heterogeneous MR patients and our approach is useful to identify known as well as novel XLMR genes, resulting in a better understanding of the clinical conditions and genetic background of XLMR, although further study is needed to assess the significance of candidate XLMR-related genes.

MATERIALS AND METHODS

Patients

We selected 144 families with at least one male having MR. 'Familial type' MR, that is more than two members of the family affected, was identified in 76 families and 'sporadic type' MR, that is only one male affected, was found in 68 families. The male probands were subjected to an aCGH using the MCG X-tiling array.[9] In 131/144 cases, conventional karyotyping was performed, and an abnormal karyotype of 46,XY,der(18)t(5;18)(p13;p11.3)pat(20/20) was detected in one case.

Cell culture

Peripheral blood samples were obtained with informed consent approved by the Institutional Review Board, National Center of Neurology and Psychiatry, Japan. Epstein–Barr virus-transformed lymphoblast cell lines (LCLs) were established from peripheral blood cells. All LCLs were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics.

aCGH using an in-house BAC array

aCGH hybridization using the MCG X-tiling array was performed as described previously with DNA extracted from sex-matched normal lymphocytes as a reference.[10] Acquired images from hybridized slides were analyzed with GenePix Pro 6.0 (Axon Instruments, Foster City, CA, USA). Fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratio across the array was zero. The thresholds for copy-number gain and loss were set at log2 ratios of 0.4 and −0.4, respectively.

High-density oligonucleotide aCGH

A genome-wide oligonucleotide aCGH was performed using 244K (Agilent Technologies, Santa Clara, CA, USA) according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner, 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).

FISH

Metaphase chromosomes were prepared from normal peripheral lymphocytes and from each of the LCLs in all family members using the standard method. Fluorescent *in situ* hybridization (FISH) analyses were performed as previously described,[10] using BAC clones located around the region of interest as probes.

Quantitative real-time reverse transcriptase-PCR

cDNAs were synthesized from total RNA extracted from LCLs established from the patients, their parents and six normal controls (three males and three females). Quantitative real-time reverse transcriptase PCR was performed with the ABI PRISM 7500 sequence detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Assays (Hs00202185_A1 FTSJ1, Hs00172868_A1 PQBP1, Applied Biosystems) according to the manufacturer's instructions. mRNA levels of the genes of interest were normalized against a housekeeping gene, *GAPDH* (Hs9999905_A1 GAPDH, Applied Biosystems), as an internal control to collect the relative expression data. Each assay was performed in triplicate for each sample.

The *androgen receptor* X-inactivation assay and late replication assay

The pattern of X-chromosome inactivation in the females was first evaluated using the *androgen receptor* X-inactivation assay[11] with minor modifications. Briefly, DNA was modified with sodium bisulfite and amplified with primers specific for a methylated or unmethylated DNA sequence at the human *androgen receptor* locus where methylation correlates with X-inactivation. Two different sized products, which were gained from the paternal and maternal alleles because of the polymorphism of the triplet repeat, were analyzed on a 3130 Genetic Analyzer (Applied Biosystems), and peak images of each PCR product were measured by GeneMapper Software v4.0 (Applied Biosystems). An imbalance of X-chromosome inactivation (skewing) was judged from the ratio between the amount of PCR product from paternal and maternal alleles. These ratios were corrected using a calculation previously described.[11]

A late replication assay was performed using a replication G-banding technique as previously reported[12] with minor modifications. Metaphase chromosomes were prepared with adjunction of 5-bromo-2-deoxiuridine in the last 6 h of cell culture after thymidine synchronization. The chromosome slides were stained with Hoechst 33258 (1 mg ml^{-1}) (Sigma, Saint Louis, MO, USA) for 5 min, and exposed to 254-nm ultraviolet light (Stratalinker UV Crosslinker 1800; Agilent Technologies) at a distance of 20 cm for 10 min after heating at 75 °C for 10 min. These chromosomes were used for FISH to estimate the ratio of inactivation of the affected X chromosome.

RESULTS

Classification of CNVs

We screened CNVs on the X chromosome in probands of 144 families with at least one affected male, by array CGH using the MCG X-tiling array to identify novel XLMR-related genes. We designed a flowchart for the classification of CNVs (Figure 1). If we detected a CNV containing known XLMR-related genes or of unknown biological or clinicopathological significance (National Center for Biotechnology

Information, http://www.ncbi.nlm.nih.gov/) and not registered in the Database of Genomic Variants (DGVs, http://projects.tcag.ca/variation/) in the male proband, we examined other family members using FISH. If the same CNV was segregated into cases of MR in the same family, it was considered a candidate for a pCNV, although CNVs observed in unaffected females in the same family or sporadic type were not excluded. Consistent with previous reports (5/108=4.6%[13] or 8/54=14.8%[14]), putative MR-associated pCNVs were detected in 10 families (6.9%, Table 1; Figure 2). The CNVs detected in five families contained known XLMR genes, whereas five candidate pCNVs seemed to be novel, although their pathogenic significance will need to be determined. The detection rates for the 'familial type' and 'sporadic type' were 7.9% (6/76) and 5.9% (4/68), respectively, suggesting that we cannot ignore the 'sporadic type' in the screening of candidate pCNVs. Each of pCNVs detected in 9 of 10 families was inherited from probands' mothers, respectively, suggesting that those CNVs were not altered through the establishment of each of the Epstein–Barr virus-transformed LCLs. In these 10 families, no abnormality was detected by conventional cytogenetics. In addition, no CNV possibly related to MR was detected in autosomes with the high-density oligonuculeotide array. Family trees of the 10 families are presented in Figure 3.

Frequent duplication at Xq28 including *MECP2*

Duplications at Xq28 including *MECP2* (OMIM 300005) were observed in 3 of 144 families (2.1%; MRYB6, MR1P3 and MR347 families in Table 1). These patients had several common phenotypes, such as severe MR, muscular hypotonia, absence of speech and recurrent respiratory infections as reported,[15–19] although the size of genes within the affected regions differed among the three families (Supplementary Figure S4a). The smallest region of overlap was ~437 kb and contained 13 genes including *L1CAM* (OMIM 08840) and *MECP2*. FISH using an *MECP2*-specific probe revealed that the mothers in all three families were carriers (Supplementary Figures S1a, S1b and S1c) and had a skewed X-inactivation pattern (Table 1) and dominant late replication pattern of the *MECP2*-duplicated allele (data not shown). In patients and the mother of family MR347, an ~182-kb deletion at Xp22.31, which contains no protein-coding gene (Supplementary Figure S4b), was detected simultaneously by aCGH analyses using an X-tilling array and Agilent oligonucleotide array, suggesting this CNV to be of unknown biological or clinical pathological significance. In addition, the CNVs at autosomal region not registered in the DGV were detected in each of the probands in three families. (Supplementary Table S2) According to ISCN 2009,[20] these CNVs are as follows: MRYB6 had arr 15q21.2 (50 711 956–50 777 075)×1; MR1P3 had arr 20p13(897 451–956 849)×1; MR347 had arr 4q13.1(61 867 547–61 924 356)×1 and arr 15q23(65 693 871–65 713 056)×1.

Aberrations at Xp11.22-p11.23 detected in two families contain known MR-related genes

We detected candidate pCNVs at Xp11.22-p11.23 in 2 of 144 families (1.4%; MR67H and MRF91 in Table 1), although the affected regions showed no overlap between these two families (Figure 4).

MRF91

The male proband (III-1) of family MRF91 showed moderate MR and speech delay. In this patient, an ~1.37-Mb duplication at Xp11.23 was detected (Figure 2). Information from the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) revealed that the duplicated region is gene-dense, and includes three known MR-related genes, *FTSJ1* (OMIM 300499), *PQBP1* (OMIM 300463) and *SYP* (OMIM 313475). No mutation was detected in those genes in the proband (data not shown). FISH revealed the duplication in the proband (III-1), mother (II-2) and his affected younger sister (III-2) (Supplementary Figure S1d). The mRNA levels of *FTSJ1* and *PQBP1* in LCLs determined by quantitative real-time reverse transcriptase-PCR were highest in the proband (Supplementary Figure S2). *SYP* mRNA levels could not be evaluated due to low expression in LCL. The X-chromosome inactivation in LCL showed a skewed pattern in the unaffected mother and random pattern in the affected sister (Supplementary Figure S3a). In addition, dup(X)(p11.23) showed a late replicating pattern in 39/50 cells (78%) of the unaffected mother and 24/50 cells (48%) of the affected sister (Supplementary Figure S3b). The high-density oligonucleotide aCGH revealed that the duplication at Xp11.23 in family MRF91 was flanked distally by a segmental duplication containing a synovial sarcoma X breakpoint families (*SSX1*, *SSX9*, *SSX4*, *SSX3* and *SSX4B*) and proximally by an additional segmental duplication containing G-antigen (*GAGE*) families (Figure 4). The aberration is as follows: arr Xp11.23 (48 089 045–49 246 795)×2 mat.

MR67H

The male proband of family MR67H showed only moderate MR. In this patient (III-1), an ~2.86-Mb deletion at Xp11.22, which has never been reported, was detected (Figure 2). Information from the UCSC genome browser revealed that the deleted region contains *SHROOM4* (OMIM 300579), reported to be a causative gene for XLMR.[21] Sample of his mother (II-4) was not available. The high-density oligonucleotide aCGH revealed that the deletion at Xp11.22 in family MR67H was also flanked distally by a sequence gap and proximally by a complex repeat-rich locus containing *SSX* families (*SSX7* and *SSX2*), *melanoma antigen* (*MAGE*) families and *X-antigen* (*XAGE*) families (Figure 4). The aberration is as follows: arr Xp11.22 (50 040 995–52 710 691)×0.

Other novel candidate pCNVs in five families

Identical complex pCNVs detected in nonconsanguineous MR22T and MRK13 families. The proband (III-1) of MR22T was diagnosed with West syndrome from electroencephalogram and showed severe MR, epilepsy, absence of speech and atrophy of the hippocampus, whereas patients (II-1, II-2) of family MRK13 manifested moderate MR, speech delay and autistic feature. Although the MR22T and MRK13 families are not consanguineous, identical duplications at the same two loci were detected: dup(X)(p22.2) containing part of *NHS* (OMIM 300457) and part of *REPS2* (OMIM 300317), and dup(X)(p21.3) containing part of *IL1RAPL1* (OMIM 300206), which was identified as an XLMR-related gene (OMIM 300143) (Supplementary Figures S4c and d). The aberration is as follows: arr Xp22.2 (16 898 131–17 635 375)×2 mat and arr Xp21.3 (28 711 594–28 812 042)×2 mat. The X-chromosome inactivation of mothers in both families showed a skewed pattern (Table 1). FISH analysis revealed that the signal for a BAC RP11-438J7 at Xp21.3 appeared separately at Xp21.3 and Xp22.2 and the signal at Xp22.2 could be detected more strongly (Figure 5).

MR1WK

The male proband (II-2) of family MR1WK showed mild MR and autism. An ~0.57-Mb duplication at Xp22.2 including a part of *FRMPD4* was detected in the patient. The high-density oligonucleotide aCGH revealed that the duplication also includes *MSL3* distal to *FRMPD4* (Supplementary Figure S4e). The aberration is as

Table 1 Summary of 10 families detected MR-associated pathogenic CNVs by array CGH analyses

Family	Family type	Locus	Aberr	BAC-based X-tiling array Start BAC	BAC-based X-tiling array End BAC	Size (Mb)	Genes	Candidate genes	Inheritance	Segregation	X-inactibvation	Phenotypes of patients
				Agilent 244K Start (bp)	Agilent 244K End (bp)	Size (bp)						
Known XLMR genes												
MRYB6	F	Xq28	Dup	RP11-846A22	CTC-384K8	0.59	22	*MECP2*	Inherited	Yes	m:80/20	Severe MR, muscular hypotonia, absent speech, reccurent respiratory infection
				152,721,477	153,436,833	715,357	31					
MR1P3	S	Xq28	Dup	RP11-846A22	RP11-119A22	0.33	10	*MECP2*	Inherited	Yes	m:70/30	Severe MR, muscular hypotonia, absent speech, reccurent respiratory infection
				152,676,750	153,158,866	482,117	19					
MR347	F	Xp22.31	Del	RP11-280C22	RP11-10G18	0.28	1	*NLGN4*	Inherited	Yes	m:96/4	Severe MR, muscular hypotonia, absent speech, reccurent respiratory infection, strong autistic feature
				6,213,159	6,395,371	182,213	0					
		Xq28	Dup	RP11-846A22	RP11-119A22	0.33	10	*MECP2*	Inherited	Yes		
				152,721,477	153,266,394	544,918	18					
MR67H	F	Xp11.22	Del	RP11-805H4	RP11-155M8	2.86	17>	*SHROOM4*	NA	NA	NA	Moderate MR
				50,040,995	52,710,691	2,669,697	>15					
MRF91	F	Xp11.23	Dup	RP11-344N17	RP11-211H10	1.37	39>	*FTSJ1, PQBP1, SYP*	Inherited	Yes	m:54/46 as:82:18	Moderate MR, speech delay
				48,089,045	49,246,795	1,157,751	>38					
Novel pCNV												
MR22T	S	Xp22.2	Dup	RP11-2K15	RP11-115I10	0.69	2	*REPS2, NHS*	Inherited	Yes	m:86/14	West syndrpme, severe MR, epilepsy, absent speech, artrophy of the hippocampus
				16,898,131	17,635,375	737,245	2					
		Xp21.3	Dup	RP11-639G8	RP11-438J7	0.23	1	*IL1RAPL1*	Inherited	Yes		
				28,711,594	28,812,042	100,449	1					
MRK13	S	Xp22.2	Dup	RP11-2K15	RP11-115I10	0.69	2	*REPS2, NHS*	Inherited	Yes	m:98/2	Moderate MR, speech delay, autistic feature
				16,898,131	17,635,375	737,245	2					
		Xp21.3	Dup	RP11-639G8	RP11-438J7	0.23	1	*IL1RAPL1*				
				28,711,594	28,812,042	100,449	1					
MR1WK	S	Xp22.2	Dup	RP11-797I1	RP11-937L19	0.57	1	*FRMPD4*	Inherited	Yes	m:59/41	Mild MR, autism
				11,680,788	12,313,191	632,404	2					
MR494	F	Xq21.1	Dup	RP11-74B21	RP11-405O21	0.33	1	*HDX*	Inherited	Yes	m:60/40 as:92:8 sb:96:4	Border-mild MR, epilepsy
				83,463,344	84,006,214	542,871	1					
MR86B	F	Xq24	Del	RP11-566B18	RP11-94I22	0.18	0	ND	Inherited	Yes	m:83/17	Moderate MR
				120,358,756	120,574,498	215,742	0					

F, familial type; S,sporadic type; Aberr, aberration; Dup, duplication; Del, deletion; NA, not available; ND, not determined.
m, X-inactivation pattern from the carrier mother; as, affected sister; sb, sister showing border of MR.

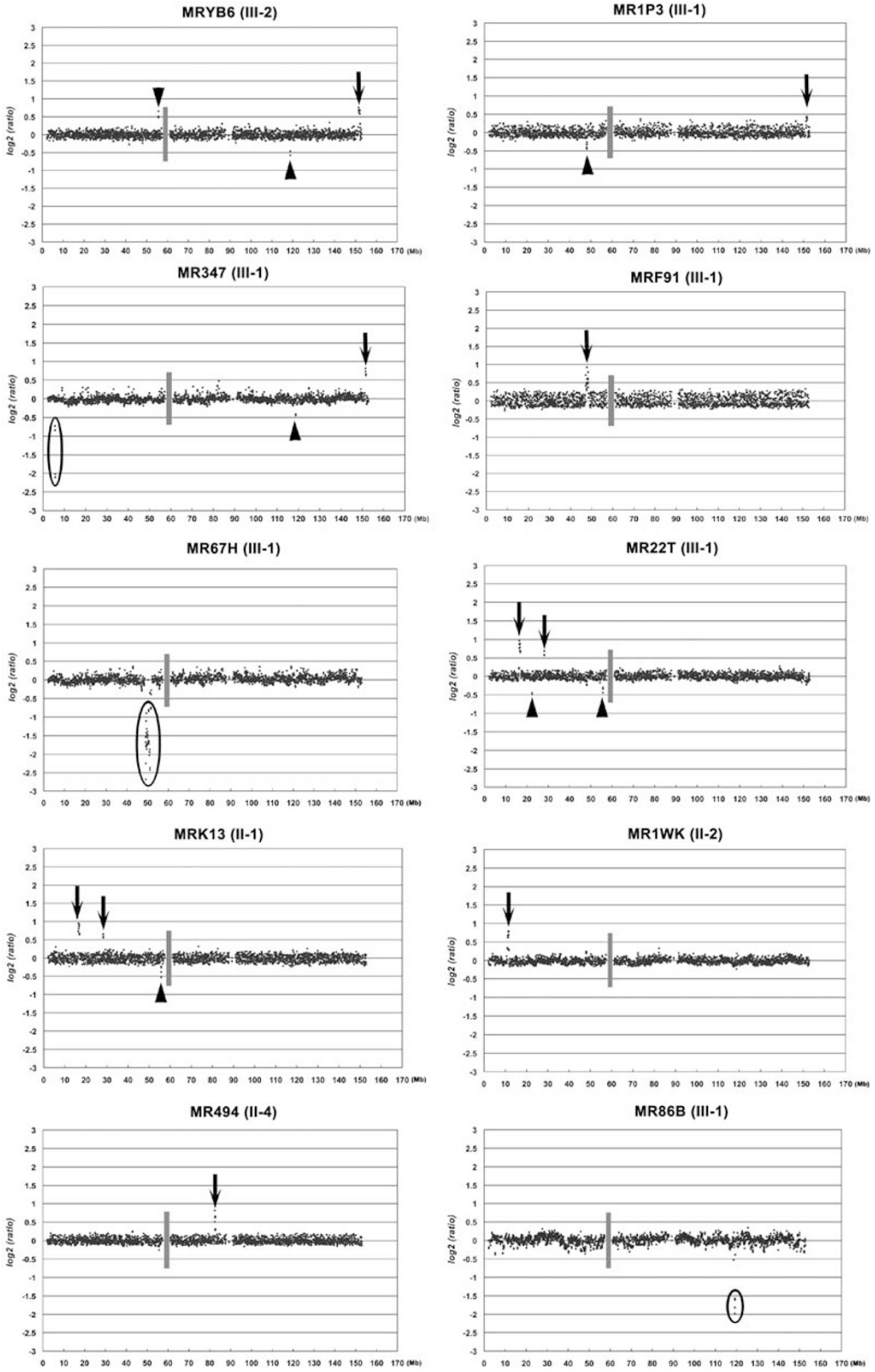

Figure 2 Results of array-CGH analysis with the X-tiling array in probands of 10 families in which candidate pCNVs were detected. Each dot represents the log2 ratio of a BAC, and arrows and circles indicate MR-associated duplications (ratio >0.4) and homozygous deletions (ratio <-0.7), respectively. Arrowheads indicate benign CNVs. The gray vertical lines represent the centromeric region for which no clones were available.

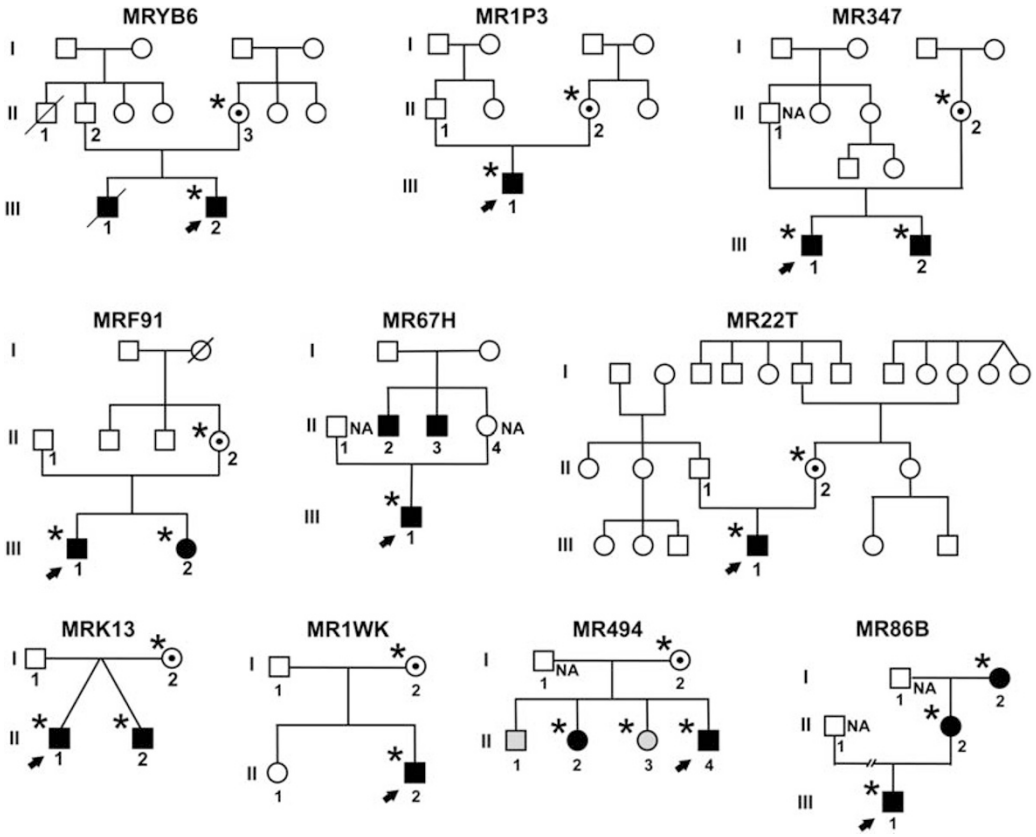

Figure 3 Pedigrees of 10 families in which probable pCNVs were detected. Closed squares and circles, gray squares and circles, and dotted circles indicate MR, borderline MR and carrier, respectively. The proband indicated by an arrow was used for CGH with the X-tiling array. Asterisks indicate persons having identical pCNVs among each family. A slash indicates that the person has died. NA, not available.

follows: arr Xp22.2 (11 680 788–12 313 191)×2 mat. FISH analysis revealed that the patient's mother (I-2) is a carrier but his unaffected sister (II-1) does not have the duplication (Supplementary Figure S1f). The carrier mother (I-2) showed a random X-inactivation pattern (Table 1).

MR494

The male proband (II-4) and his sister (II-2) were affected by moderate MR, and his brother (II-1) and another sister (II-3) had borderline MR (Figure 3). All his siblings had localization-related epilepsy in childhood. In the proband, an ~0.33-Mb duplication including *HDX* was detected at Xq21.1 (Supplementary Figure S4f). The aberration is as follows: arr Xq21.1 (83 463 344–84 006 214)×2 mat. FISH revealed that the unaffected mother (I-2), affected sister (II-2) and sister (II-3) with borderline MR had the same duplication, whereas the brother with borderline MR did not (Supplementary Figure S1g). The X-chromosome inactivation of the mother (I-2) had a random pattern and affected sister (II-2) and another sister (II-3) with borderline MR had a skewed pattern (Table 1).

MR86B

All three patients in the MR86B family had moderate MR. In the male proband (III-1), an ~0.18-Mb deletion was detected at Xq24 by aCGH (Figure 2). The aberration is as follows: arr Xq24 (120 358 756–120 574 498)×0 mat. This deletion contains no protein-coding genes but eight human expression sequence tags: DA381697, CB043836. CB043837, AW193789, AW894827, BF374258, AA191179 and AA789076 (Supplementary Figure S4g). The deletion was inherited from his affected grandmother and his affected mother (Supplementary Figure S1h). The X-chromosome inactivation showed a skewed pattern in the affected mother (II-2) and grandmother (I-2) (Table 1).

Detection of nine benign CNVs

We detected possible benign CNVs, which may not be associated with the MR phenotype, in nine regions based on our flowchart (Supplementary Table S1). Among them, one region containing several genes at Xp22.2 had not been registered in the DGV, and another region at Xq22.1 contains *protocadherin 19* (*PCDH19*, (OMIM 300460)), although its mutations have been reported to be related to female patients with MR (EFMR (OMIM 300088)).[22]

DISCUSSION

A duplication at Xq28 containing *MECP2* is one of the most common genomic rearrangements in neurodevelopmentally delayed male.[15] In this study, the duplication at Xq28 involving *MECP2* was detected in Japanese patients at high frequency (3/144=2.1%) compared with reported cases in Western countries (1/108=0.9%[13] or 1/54=1.9%[14]). The patients manifested several common phenotypes such as severe MR, muscular hypotonia, absence of speech and recurrent respiratory infections as reported.[15–19] Mapping at dup(X)(q28) of our three families indicated that the smallest region of overlap contained thirteen genes including *L1CAM* and *MECP2* (Supplementary Figure S4a), suggesting that these genes contribute to their

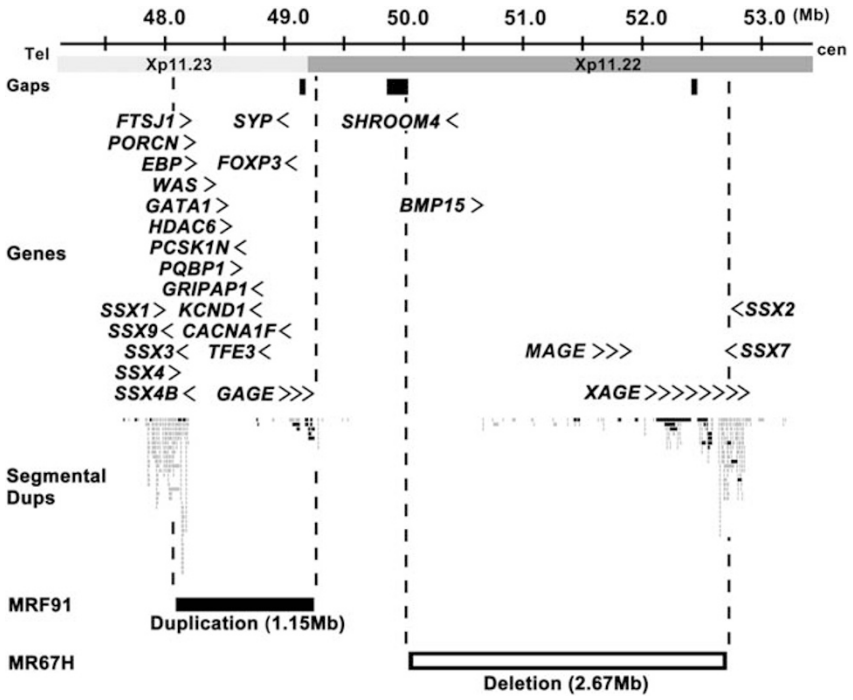

Figure 4 Mapping of aberrations at Xp11.23-p11.22 detected in families MRF91 and MR67H. Disease-associated and copy-number-sensitive genes (not drawn to scale) are described by chevrons. Gaps in the genome assembly, segmental duplications (Dups) are shown. Filled bars and gray bars indicate >99% similarity and 90–99% similarity, respectively, in segmental duplications. High-density oligonucleotide array (Agilent 244K) revealed an ~1.15-Mb duplication at Xp11.23 in MRF91 (filled bar) and an ~2.67-Mb deletion at Xp11.23-p11.22 in MR67H (open bar).

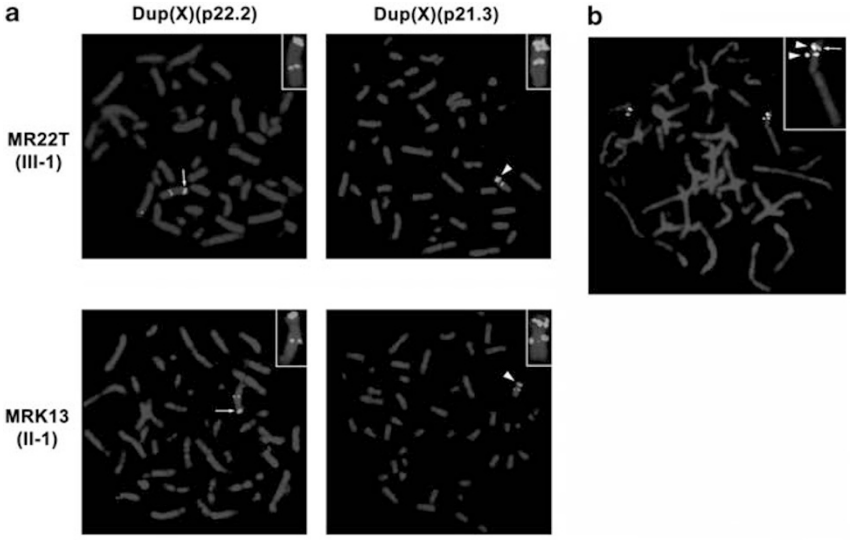

Figure 5 Duplicon at Xp21.3 were observed at Xp22.2 by FISH. (**a**) Representative results of FISH in probands of MR22T (upper) and MRK13 (lower). FISH using the clone RP11-2K15 at Xp22.2 (left column) and the clone RP11-438J7 at Xp21.3 (right column) showed strong green signals (arrows) and separate green signals (arrowheads), respectively, and the clone RP11-13M9 at Xq13.2 shows red signals as a reference in both experiments. An enlarged image of chromosome X is shown in the upper right insets in each panel. (**b**) Representative results of FISH in the patient's mother (I-2) in MRK13 with elongated metaphase chromosomes prepared as described elsewhere.[44] The clone RP11-2K15 at Xp22.2 and the clone RP11-438J7 at Xp21.3 demonstrated strong signals (red) and separate signals (green), respectively, in one allele. An enlarged image of the duplicated allele is shown in the upper right inset, indicating that the duplicated sequence at Xp22.2 was inserted in close proximity (arrow), whereas the duplicated sequence at Xp21.3 inserted into the duplication at Xp22.2 together with the original Xp21.3 (arrowheads). A full color version of this figure is available at *The Journal of Human Genetics* journal online.

phenotypes. Carvalho *et al.*[23] proposed the presence of low-copy repeats in the vicinity of the *MECP2* gene to be involved in the rearrangement including *MECP2*. In our cases, the distal breakpoints of all duplications were located on segmental duplications (Supplementary Figure S4a). In family MR347, interestingly, an ~182-kb deletion at Xp22.31 together with dup(X)(q28) were also detected in

two patients (III-1, III-2) showing strong autistic features and their carrier mother (II-2) (Supplementary Figure S4b). Although no protein-coding genes are located within the del(X)(p22.31), *NLGN4X* (OMIM 300427), which is known to be associated with autism,[24] is located near this region (~55 kb from the distal breakpoint), suggesting that this deletion may work as modifier for their phenotypes. In all mothers of the three families, the duplicated allele showed a late replication pattern dominantly (data not shown), indicating that they are not phenotypes due to the X-inactivation pattern. On the other hand, CNVs at autosomal region not registered in the DGV were detected in each of probands in three families (Supplementary Table S2). These CNVs contain protein-coding genes (*LPHN3*, *KIAA1370*, *MAP2K5* (OMIM 602520), *RSPO4* (OMIM 610573)). Although *RSPO4* are related to anonychia (OMIM 206800),[25,26] the proband of MR1P3 family having the deletion at this region has not shown anonychia and phenotypes have shown no difference among these three probands, suggesting that these CNVs were benign CNVs that have not been associated with the disease.

In the MRF91 family, an ~1.16-Mb duplication at Xp11.23 including *FTSJ1*, *PQBP1* and *SYP* was detected in the male proband (III-1), his affected younger sister (III-2) and their unaffected mother (II-2) (Figure 2; Supplementary Figure S1d). Duplications in the same region were reported in two males and six females of 2400 subjects (0.33%) with MR, speech delay and electroencephalographic anomalies.[27] Although electroencephalographic recordings have not been examined in the patients of family MRF91, phenotypic similarity, such as moderate MR and speech delay, among reported cases and our cases suggests genes located within this duplicated region to be associated with those phenotypes. This hypothesis is supported by higher expression of *FTSJ1* and *PQBP1* mRNAs in the male proband (III-1) compared with the carrier mother (II-2) and the affected sister (III-2) and in MRF91 family members having dup(X)(p11.23) compared with normal controls (Supplementary Figure S2). As dup(X)(p11.23) in both the sister (III-2) and mother (II-2) showed a late replicating pattern (Supplementary Figure S3), expression of *FTSJ1* and *PQBP1* with the duplication seems to be predominantly repressed by X-inactivation. However, *FTSJ1* expression in the affected sister (III-2) was lower than that in the carrier mother (II-2) but *PQBP1* expression showed the reverse (Supplementary Figure S2), suggesting that expression levels might not be perfectly reflected by X-inactivation status. *PQBP1* levels of proband (III-1), sister (III-2) and mother (II-2) with dup(X)(p11.23) were higher than the average +3 s.d. of 10 healthy controls, suggesting that *PQBP1* expression may be influenced by the duplication more strongly at least in LCLs. Nonsense mutations in *PQBP1* were detected in patients with MR and microcephaly.[28] It is noteworthy that our cases showed macrocephaly, suggesting that this phenotype results from increased *PQBP1* expression. In addition, excessive action of *PQBP1* has been shown to cause neuronal dysfunction,[29–33] indicating increased expression of *PQBP1* through duplication to be involved in MR with macrocephaly.

In the proband (III-1) of family MR67H, a novel ~2.67-Mb deletion at Xp11.23-p11.22 harboring *SHROOM4* (Figure 2). A missense exchange of *SHROOM4* was reported to segregate with Stocco dos Santos XLMR syndrome (OMIM 300434) in a large four-generation pedigree.[21] The affected males in the reported family presented with severe MR, delayed or no speech, seizures and hyperactivity. Our patient with null *SHROOM4* showed only moderate MR, suggesting that the missense exchange detected in Stocco dos Santos XLMR syndrome contributes to the pathogenesis of MR together with other phenotypes in a gain-of-function manner. Although genetic status in other members of the family was not investigated because of a lack of available materials, two of the proband's maternal uncles (II-2, II-3) had MR (Figure 3), suggesting that this deletion was inherited from the proband's mother (II-4) and probably contributes to MR. An oligonucleotide-array analysis revealed that breakpoints of dup(X)(p11.23) and del(X)(p11.22) were mapped within segmental duplications containing *SSX* genes, *GAGE* genes and *XAGE* genes and sequence gap, respectively (Figure 4). Previous reports[13,27] suggested that CNVs at Xp11.22-p11.23 were associated with flanking segmental duplications. In addition, broken forks are the precursor lesions directly processed into segmental duplications in yeast[34] and Fork Stalling and Template Switching (FoSTeS) has been proposed as a replication-based mechanism that produces nonrecurrent rearrangements potentially facilitated by the presence of segmental duplications.[35] Thus, the del(X)(p11.22) containing *SHROOM4* might occur through a segmental duplication-dependent manner.

We detected five novel candidate pCNVs, which have not been identified by similar screenings,[13,14] on the X chromosome in probands of five families, and each of their mothers had aberrations concordant with that detected in the proband. In MR22T and MRK13 families, we detected two identical duplications at Xp22.2 and Xp21.3 (Figure 2). FISH revealed that the duplicated sequence at Xp21.3 exists near the duplicated sequence at Xp22.2 (Figure 5), suggesting that these duplications were related to each other and occurred simultaneously. Thus, although large CNVs at Xp21.3 are registered in DGV (Supplementary Figure S4d), the duplication detected in this study is different from CNVs. The duplications showed complicated genomic rearrangements and the involvement of parts of genes (*REPS2*, *NHS* and *IL1RAPL1*). *IL1RAPL1* was identified as an XLMR gene. *REPS2* is associated with a small G protein and shows strong expression in brain tissue (LSBM, http://www.lsbm.org/index.html). As alterations in signaling pathways involving the Rho family of small GTPases contribute to both syndromic and nonsyndromic MR disorders[36] and mutation in the small GTPase gene *RAB39B* (OMIM300774) were identified in two MR patients,[37] it is possible that deregulated expression of *REPS2* contributes to MR. Protein-truncation mutations in *NHS* have been identified in patients with Nance-Horan syndrome (OMIM 302350), an X-linked developmental disorder characterized by congenital cataracts, dental anomalies, facial dysmorphism and MR in some cases. As our patients in both families did not have cataracts or dental anomalies, the genomic rearrangement involved in *NHS* may not affect the function of this gene. Although the characteristic CNVs observed in two patients of two unrelated families were identical, the severity of MR was different between patients: one patient had severe MR but the other had moderate MR, suggesting this complicated genomic rearrangement to vary in penetrance among individuals. It has not yet been clarified whether the complex CNV was transmitted from a single founder or occurred independently. Further analysis such as genomic DNA sequencing and/or screening among different ethnic groups will be needed to disclose the full details of it.

In family MR1WK, an ~632-kb duplication at Xp22.2 was detected in the affected son (II-2) and unaffected mother (I-2), indicating that it was segregated with the disorder (Figure 2; Supplementary Figure S1f). The duplicated region includes *MSL3* and a part of *FRMPD4* (Supplementary Figure S4e). *MSL3* was identified as a human homolog of *Drosophila* male-specific lethal 3 (msl3) and expressed ubiquitously in adult tissues.[38] The expression level of *MSL3* was not increased in the proband with the duplication (data not shown). *FRMPD4* showed a high expression level specifically in human brain tissue (LSBM), and contains one PDZ domain, a protein-interaction domain frequently found in multidomain scaffolding proteins,

although its function has not been identified. PDZ-domain-containing scaffolds also have a role in the dynamic trafficking of synaptic proteins by assembling cargo complexes for transport by molecular motors.[39] Although the level of *FRMPD4* could not be identified because of its low expression in LCL, it is possible that the duplication of disrupted *FRMPD4* caused the mild MR and autistic features in our case.

In family MR494, an ~542-kb duplication at Xq21.1 included *HDX* encoding a protein containing 2 homeobox DNA-binding domains whose function is unknown (Supplementary Figure S4f). In humans, mutations of homeobox genes, shown as *ARX* (OMIM 300382), *PAX6* (OMIM 607108) and *NKX2-1* (OMIM 600635), expressed in the forebrain have been shown to result in MR, epilepsy or movement disorder.[40] Although this duplication was not detected in the oldest brother showing borderline MR and the affected localization-related epilepsy as well as other siblings, the HDX may be relevant to MR. The distal breakpoint located in the genomic region within segmental duplications according to the database (Supplementary Figure S4f), suggesting segmental duplications to be involved in the generation of the duplication.

In family MR86B, we detected an ~215-kb deletion at Xq24 containing no protein-coding gene but eight human expression sequence tags whose sequence is conserved in only primates (UCSC genome browser) (Supplementary Figure S4g), suggesting that a defect of them may contribute to the disease. In addition, *CUL4B* (OMIM 300304) and *GRIA3* (OMIM 305915), which were reported as XLMR-associated genes,[41,42] are located around the region involved (Supplementary Figure S4g). It is possible that the deletion alters expression levels through some mechanism, such as a defect in binding of transcription factor(s) and alteration of the chromatin structure.

In this study, we detected nine benign CNVs thought not to be associated with MR on the basis of our flowchart (Supplementary Table S1). Among them, two CNVs were ruled out because of no segregation with MR in the family, although one contains *PCDH19*, a known XLMR gene, and another has not been recorded in the DGV. Therefore, if MR is caused by environmental factors in affected family members not having CNVs, it is possible that these CNVs are relevant to MR. Interestingly, mutation in *PCDH19* caused MR in females, but not in males,[22] and a duplication of *PCDH19* was detected in male patients in our study, suggesting that this duplication may contribute to MR in males. Recently, Girirajan *et al.*[43] hypothesized that genomic alterations, such as large CNVs observed second alterations other than the risk CNVs, serve as 'second hits' that convert the risk CNV from a risk factor to a determinant or modifier of the developmental phenotype. Therefore, it is possible that nine CNVs, which we considered as benign CNV, may be risk CNVs, which need second-site genomic events to produce a severe phenotype. Further detailed analyses including whole-genome sequencing will be needed to clarify this possibility.

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APPENDIX

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