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 / Agilent 244K Start (bp)	BAC-based X-tiling array End BAC / Agilent 244K End (bp)	Size (Mb) / Size (bp)	Genes	Candidate genes	Inheritance	Segregation	X-inactibvation	Phenotypes of patients
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.

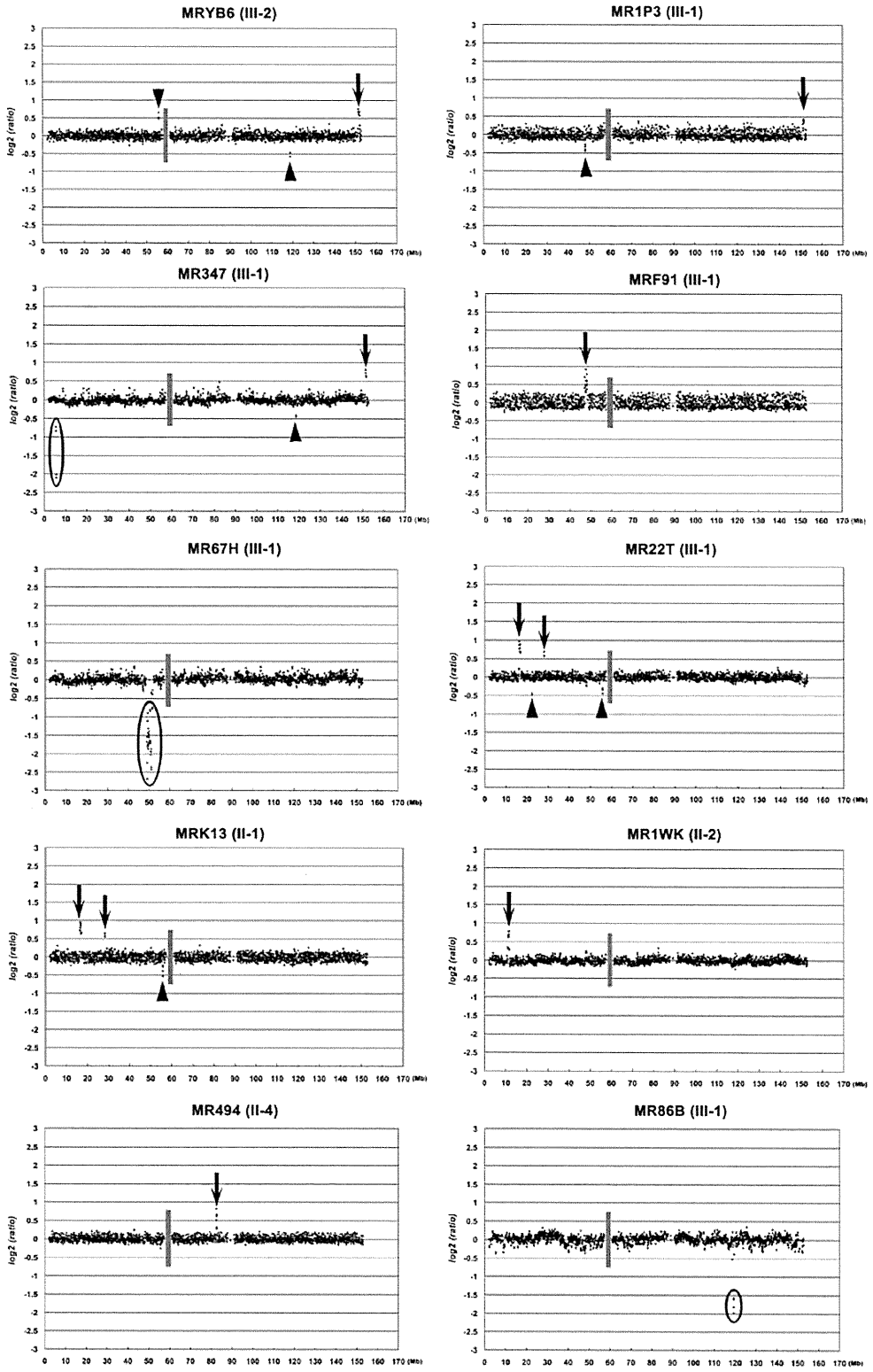

Figure 2 Results of array-CGH analysis with the X-tiling array in probands of 10 families in which candidate pCNVs were detected. Each dot represents the log2 ratio of a BAC, and arrows and circles indicate MR-associated duplications (ratio >0.4) and homozygous deletions (ratio <−0.7), respectively.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

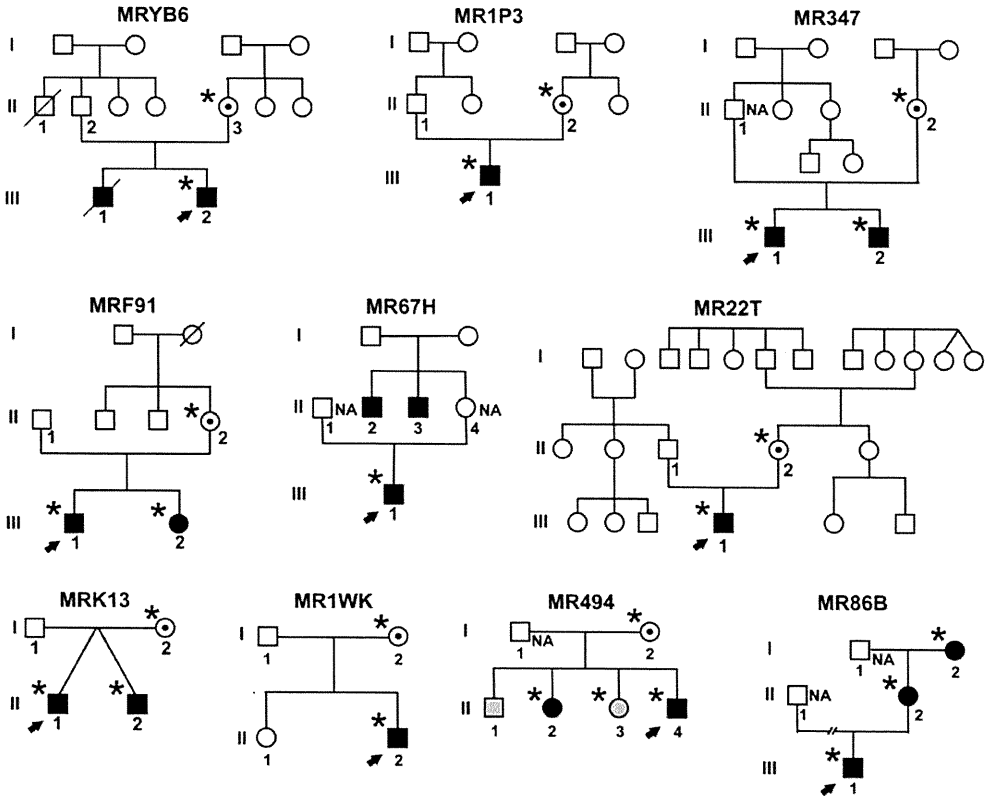

Figure 3 Pedigrees of 10 families in which probable pCNVs were detected. Closed squares and circles, gray squares and circles, and dotted circles indicate MR, borderline MR and carrier, respectively. The proband indicated by an arrow was used for CGH with the X-tiling array. Asterisks indicate persons 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

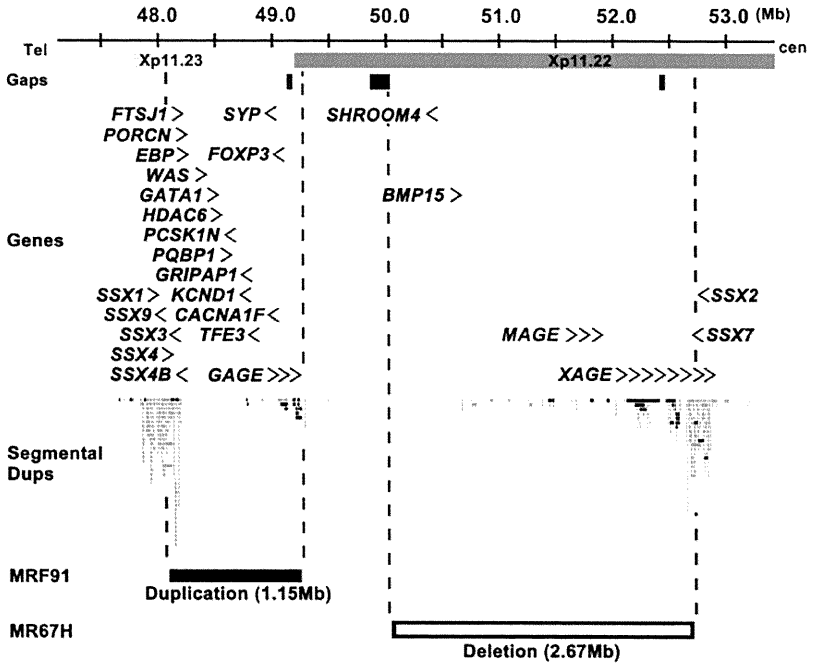

Figure 4 Mapping of aberrations at Xp11.23-p11.22 detected in families MRF91 and MR67H. Disease-associated and copy-number-sensitive genes (not drawn to scale) are described by chevrons. Gaps in the genome assembly, segmental duplications (Dups) are shown. Filled bars and gray bars indicate >99% similarity 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).

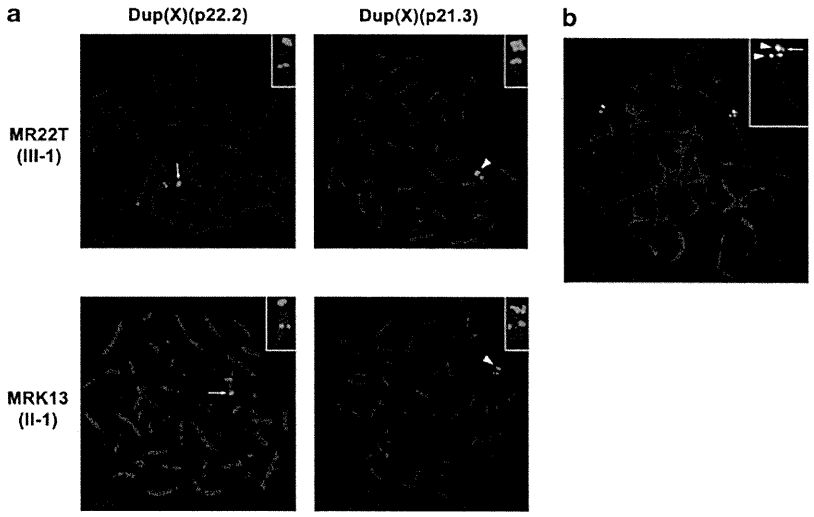

Figure 5 Duplicon at Xp21.3 were observed at Xp22.2 by FISH. (**a**) Representative results of FISH in probands of MR22T (upper) and MRK13 (lower). FISH using the clone RP11-2K15 at Xp22.2 (left column) and the clone RP11-438J7 at Xp21.3 (right column) showed strong green signals (arrows) and separate 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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SHORT COMMUNICATION

Concomitant microduplications of *MECP2* and *ATRX* in male patients with severe mental retardation

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Investigations of chromosomal rearrangements in patients with mental retardation (MR) are particularly informative in the search for genes involved in MR. Here we report a family with concomitant duplications of methyl CpG binding protein 2 (*MECP2*) at Xq28 and *ATRX* (the causative gene for X-linked alpha thalassemia/mental retardation) at Xq21.1 detected by array-comparative genomic hybridization. The alterations were observed in a 25-year-old man who inherited them from his mother, who showed a normal phenotype and completely skewed X-chromosome inactivation, and also in his cousin, a 32-year-old man. The proband and his cousin showed severe MR, muscular hypotonia, recurrent respiratory infections and various other features characteristic of *MECP2* duplication syndrome. However, the proband also had cerebellar atrophy never reported before in *MECP2* duplication syndrome, suggesting that his phenotypes were modified through the *ATRX* duplication in an additive or epistatic manner.
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Keywords: array CGH; ATRX; duplication; MECP2; X-linked mental retardation

Duplication at Xq28 involving methyl CpG binding protein 2 (*MECP2*) has been detected at high frequency (1–2%) in males with unexplained X-linked mental retardation (XLMR).[1,2] *MECP2* duplication syndrome is now recognized as a clinical entity showing severe MR, muscular hypotonia, absence of speech, a history of recurrent infection and mild dysmorphic features.[3] In the course of a program to screen possible patients with XLMR for copy-number aberrations by array-comparative genomic hybridization (aCGH) using a bacterial artificial chromosome (BAC)-based X-tiling array (MCG X-tiling array),[2,4] we detected an ~0.4-Mb duplication at Xq28 involving *MECP2* together with an ~0.3-Mb duplication at Xq21.1 that included *ATRX*, the causative gene for ATR-X (X-linked alpha thalassemia/mental retardation) syndrome, in a 25-year-old man and his cousin, a 32-year-old man (Figure 1a).

The proband (III-1, Figure 1b) was born at 41 weeks after an uneventful pregnancy as the first child of non-consanguineous healthy parents. At birth, his weight and occipital–frontal circumference (OFC) were 3280 g (±0 s.d.) and 33.5 cm (0.3 s.d.), respectively. He was developmentally retarded: first smiling at 3 months, holding up his head at 5 months, rolling over at 7 months, sitting by himself at 12 months and crawling at 13 months. At 25 years, his height, weight and OFC were 160.8 cm (−1.7 s.d.), 50 kg (−1.2 s.d.) and 56.3 cm (−0.9 s.d.), respectively. The proband exhibited hypertelorism, microcephaly and synophrys (Figure 1c). At 28 years, magnetic resonance imaging (MRI) showed cerebral atrophy, cerebellar atrophy and a thin corpus callosum (Figure 1d). He could walk and communicate until he was 14 years old, but became unable to do either of this after developing epilepsy. At the age of 4 years and 10 months, his total Developmental Quotient was 22, calculated by using the Kyoto Scale of Psychological Development. A blood investigation showed that his IgA level was low. The HbH inclusion body that is detected frequently in patients with *ATRX* mutation was not found by brilliant cresyl staining. His younger brother (III-2) had intrapartum asphyxia and two maternal uncles (II-3, II-4) died immediately after birth.

The cousin of the proband (III-3) was born in 41 weeks after an uneventful pregnancy to non-consanguineous healthy parents by normal delivery. At birth, his weight and OFC were 2850 g (−1.2 s.d.) and 37 cm (+2.4 s.d.), respectively. He was characterized by macrocephaly. He had started smiling at 2–3 months, holding up his head at 4 months, sitting by himself at 12 months and walking at 40 months. At 32 years of age, his height, weight and OFC were in the normal range (164.5 cm, −1.1 s.d.; 57 kg, −0.5 s.d.; 59.4 cm, +1.8 s.d.). Information on his Developmental Quotient was unavailable. A blood investigation showed that his IgA level was low. He had been affected

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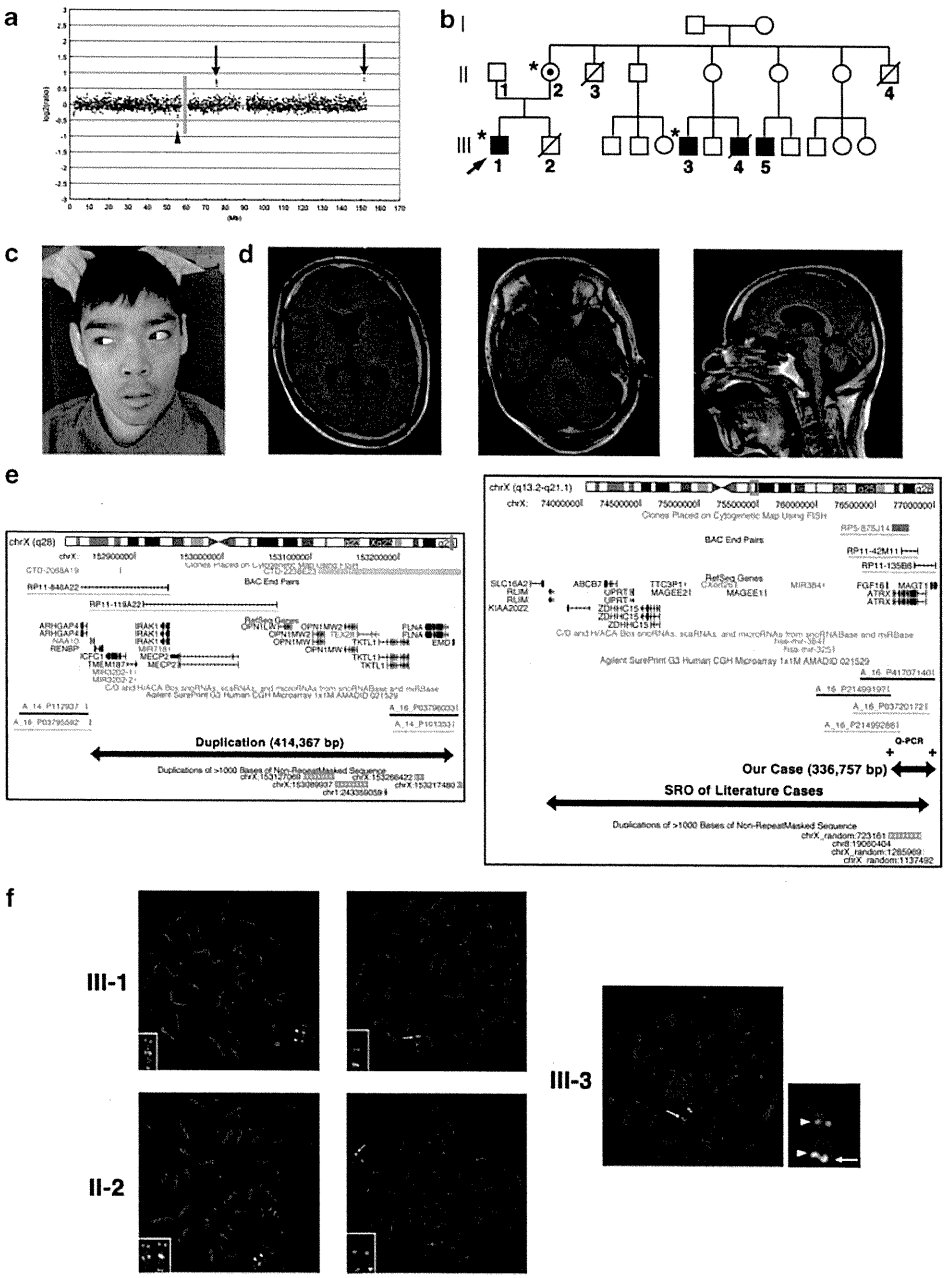

Figure 1 (**a**) Profile of the copy-number ratio on chromosome X in the proband (III-1) detected with aCGH using an MCG X-tiling array. Each dot represents the test/reference value after normalization and $\log_2$ transformation in each BAC clone, and arrows indicate duplications (ratio>0.4). The gray vertical lines represent the centromeric region for which no clones were available. Arrowheads indicate benign CNVs (Supplementary Table 1). (**b**) Three-generation genealogy of the studied family. Closed squares and circles and dotted circles indicate MR and carriers, respectively. The proband (III-1) indicated by an arrow was used for aCGH with the X-tiling array. Asterisks indicate persons having the duplications at Xq21.1 and Xq28. A slash indicates a death. (**c**) The proband (III-1) at 27 years showed hypertelorism, microcephaly and synophrys. (**d**) Brain MRI findings of the proband (III-1) at age 27. Coronal (left and middle) and (right) sagittal T1w sequences show cerebral atrophy, cerebellar atrophy and a thin corpus callosum. (**e**) Mapping of the duplications at Xq28 (left) and Xq21.1 (right) on the basis of the UCSC Genome Browser according to NCBI Build 36.1, March 2006, hg18 (http://www.genome.ucsc.edu/). A chromosome ideogram is presented. The track setting in the UCSC genome browser was set up to look for 'Base Positions', 'FISH Clones', 'BAC End Pairs', 'RefSeq Genes', 'sno/miRNA', 'Agilent Array' and 'Segmental Duplications'. Underlines below the BAC clone ID and oligonucleotide probe ID show a high (green bars) and normal (black bars) ratio detected with the MCG X-tiling array and Agilent array 244 K. Plus signs in the right panel indicate the duplication at Xq21.1 confirmed by quantitative genomic PCR. Duplicated regions in our case and the smallest region of overlap in reported cases[9] in the right panel are indicated with closed arrows. (**f**) In the proband (III-1) and the carrier mother (II-2), representative results of FISH using the clone RP11-42M11 at Xq21.1 (left) and the clone RP11-119A22 at Xq28 (right) showed separate green signals (arrowheads) and strong green signals (arrows), respectively. The red signals are of clone RP11-16H4 at Xp22.12 (left) RP11-13M9 at Xq13.2 (right) as a reference. Enlarged images of chromosome X are shown in the lower left insets in each panel. Similarly, in the affected proband's maternal cousin (III-3), representative results of FISH demonstrated separate red signals of the clone RP11-42M11 at Xq21.1 and strong green signals of the clone RP11-119A22 at Xq28. An enlarged image of chromosome X is shown in the lower right panel, indicating that the duplicated sequence at Xq21.1 inserted into the duplication at Xq28 together with the original Xq21.1 (arrowheads), whereas the duplicated sequence at Xq28 was inserted in close proximity (arrow). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

by pneumonia frequently since 3 months after birth. No MRI analysis had been performed. His younger brother (III-4) died because of disseminated intravascular coagulation at the age of 29, and his other cousin (III-5) shows a similar clinical manifestation to the proband.

On the basis of the results of precise mapping with an oligonucleotide array (Agilent array 244K, Palo Alto, CA, USA; data not shown), these aberrations are as follows: arr Xq21.1 (76646979–76983735)×2, arr Xq28 (152847991–153262357)×2 (Figure 1e). Although some copy-number variants (CNVs) were detected in other regions simultaneously, all of them have been registered in the Database of Genomic Variants (http://projects.tcag.ca/variation/ assembly, March 2006, Supplementary Table 1) and in part in our CNV database (MCG CGH database, http://www.cghtmd.jp/CNVdatabase). Subsequent real-time quantitative genomic PCR (qPCR) using primer sets recognizing around dup(X)(q21.1) (Supplementary Table 2) narrowed down dup(X)(q21.1) to between positions 76646868 and 76973049, including all of *ATRX* and part of *MAGT1* (Figure 1d). Fluorescence *in situ* hybridization (FISH) detected these duplications in the proband's unaffected mother (II-2) and his affected maternal male cousin (III-3) (Figures 1b and f), indicating maternally inherited duplications in these patients. In addition, the duplicated segment at Xq21.1 inserted into the duplicated region at Xq28, by contrast the segment at Xq28 was duplicated in tandem (Figure 1f). Our finding that the mother, a presumptive obligate carrier, had completely skewed X inactivation (dup(X):X=50:0) in a lymphoblastoid cell line , as shown by the androgen receptor X-inactivation assay described previously[5] and a late replication assay[6] with FISH (Supplementary Figure 1), supported our assumption that skewed X-chromosome inactivation appears to be characteristic of carriers of *MECP2* duplication such as other reported cases.[3]

The two affected men showed severe MR, muscular hypotonia, recurrent respiratory infections and various other features characteristic of *MECP2* duplication syndrome (Table 1). Moreover, they did not show short stature, hypoplastic genitalia and early life feeding issues, which were reported to be characteristic of MR in patients with duplications encompassing *ATRX* (Table 1).[9] The smallest region of overlap (SRO) of the reported *ATRX* duplication cases contains 11 genes, including *ATRX* and two miRNAs,[9] whereas the duplicated region of the present family includes only *ATRX* (Figure 1e), suggesting that genes other than *ATRX* within the SRO contribute to phenotypes observed in previously reported cases (Table 1).[9]

ATRX interacts with MECP2 *in vitro* and colocalizes at pericentromeric heterochromatin in mature neurons of the mouse brain.[10] Recently, it was reported that ATRX, MECP2 and cohesin cooperate to silence a subset of imprinted genes in the postnatal mouse brain.[11] Those experimental findings suggest that abnormally expressed ATRX with MECP2 through their simultaneous duplications may modify the phenotypes usually observed in *MECP2* duplication syndrome. Although our patients showed neither notably different nor more severe phenotypes compared with reported patients with *MECP2* duplication syndrome, the proband was found to have cerebellar atrophy by MRI (Figure 1e), which has never been reported before in *MECP2* duplication syndrome.[1,3] It is possible that these phenotypes in the proband were modified through ATRX duplication in an additive or epistatic manner.

The mutations in *ATRX* give rise to changes in the pattern of methylation of several highly repeated sequences, including the ribosomal DNA (rDNA) arrays[12] and significantly altered mRNA expression in four ATRX targets (*NME4*, *SLC7A5*, *RASA3* and *GAS8*) relative to normal controls.[13] Although a Southern blot hybridization method reported previously[12] showed no change in the pattern of methylation at rDNA arrays compared with normal controls (Figure 2a), quantitative RT-PCR revealed that the expression of *ATRX* was upregulated in the present cases. Although *SLC7A5* expression showed no previous change compared with that in the healthy control (Figure 2b) and the expression of *GAS8* was too low for quantitative RT-PCR (data not shown), the expression of *NME4* and *RASA3* was similar to that in the patients with *ATRX* mutations. The alteration to the expression may be influenced by *MECP2* duplication or additive/epistatic effect between *ATRX* and *MECP2* duplication.

Table 1 Phenotype comparisons between our cases and MECP2 duplication syndrome patients or patients with *ATRX* duplication

Phenotype	*MECP2* duplication syndrome[3,7,8]	*ATRX* duplication cases[9]	Our Cases III-1	Our Cases III-3
Mental retardation	118/119	11/11	+	+
Hypotonia	86/93	7/11	+	+
Absent speech	63/72	NA	+	+
Lack ambulation	20/71	NA	+	+
Recurrent infection	82/111	6/7	+	+
Breathing abnormalities	6/18	NA	–	+
Stereotyped hand movements	15/33	NA	+	+
Autistic features/autism	13/17	NA	+	+
Epilepsy	57/110	NA	+	+
GU abnormalities	29/67	7/10 (Hypoplastic genitalia)	–	+ (Bladder distention)
Death before 25 years	25/66	NA	–	–
Spasticity	42/71	NA	–	+
Ataxia	20/37	NA	+	+
GER	15/25	NA	–	+
Swallowing difficulty	23/45	NA	–	+
IPO or constipation	25/33	NA	–	–
IgA deficiency	4/10	NA	+	+
Short stature	NA	11/11	–	–
Early life feeding issues	NA	7/9	–	–
Failure to thrive	16/31	7/9	–	+
Broad thorax	NA	4/4	–	+
Pectus excavatum	NA	3/7	–	–
Short neck	NA	4/8	–	–
Simian crease	NA	4/5	–	–
Digital findings	22/52	6/7	–	–
Microcephaly	24/71	8/11	+	–
Hypertelorism	8/72	2/6	+	+
Epicanthal folds	4/72	6/8	–	–
Down-slanted palpebral fissures	NA	1/8	–	–
Ptosis	2/72	6/9	–	–
Flat nasal bridge	15/72	9/10	–	+
Down-turned corners of the mouth	NA	8/10	–	–
High-arched palate	3/72	4/4	–	–
Micro/retrognathia	NA	4/7	–	–
Low set ears	NA	4/10	–	–
Simple ears	NA	2/10	–	–
Cryptorchidism	2/4	9/10	+	–
Impaired social interaction	NA	5/6	+	+

Abbreviations: ATR-X, the causative gene for X-linked alpha thalassemia/mental retardation; GER, gastroesophageal reflux; GU, genito-urinary system; IPO, intestinal pseudo-obstruction; *MECP2*, methyl CpG binding protein 2; NA, not available.

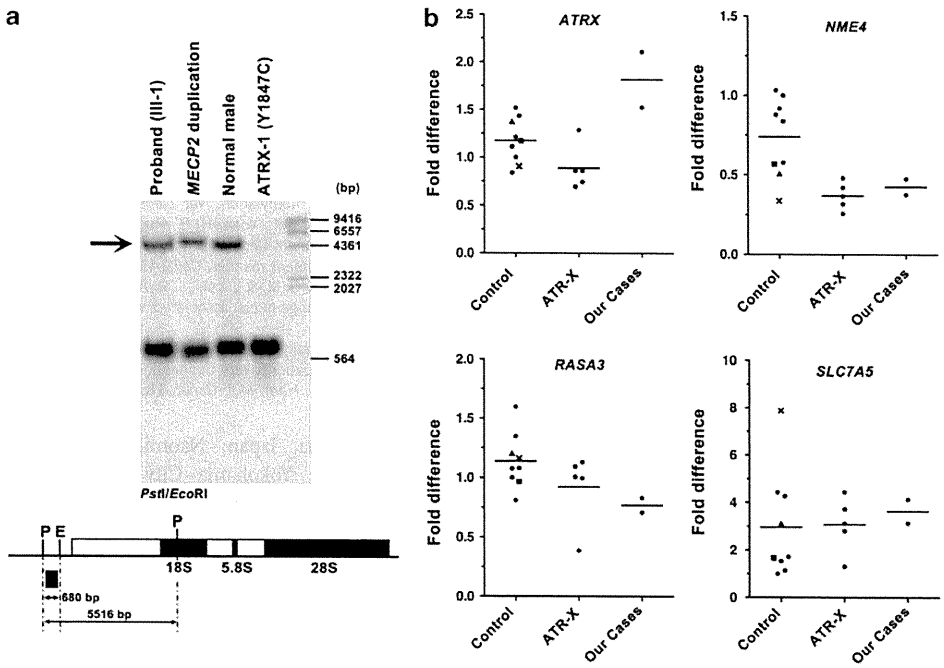

Figure 2 (**a**) Investigation of the methylation pattern of human rDNA repeats using Southern blotting. Genomic DNA from the lymphoblastoid cell line of our proband (III-1) with the *ATRX* duplication and the *MECP2* duplication, an MR male patient with *MECP2* duplication, an unrelated normal male, and an ATR-X patients with a missense mutation resulting in Y1847C in ATRX (Supplementary Table 3). DNA samples were digested with *Pst*I followed by the methylation-sensitive enzyme *Eco*RI. Hybridization is shown for probes corresponding to the region between restriction sites of the two enzymes in the 3′ end of the non-transcribed spacer. The methylated, uncut band is indicated (arrow). A restriction map of part of the rDNA repeat unit shown with the 18S, 5.8S and 28S genes in order and transcribed spacer as filled and open boxes, respectively, represents the sites for *Pst*I (P) and *Eco*RI (E). A black bar indicates the probe for the Southern hybridization. The size of the DNA segment resulting from the restriction enzymes is represented by closed arrows. (**b**) Real-time quantitative RT-PCR analysis of the mRNA expression of *ATRX* and three *ATRX* target genes (*NME4*, *RASA3* and *SLC7A5*) but not *GAS8*, the expression of which was too low to be estimated, in lymphoblastoid cells of our two patients, ATR-X patients whose *ATRX* mutations were identified through routine screening in a set of known XLMR genes by the Japanese Mental Retardation Consortium (unpublished data, n=5; Supplementary Table 3) and controls, including six healthy samples, the proband's parents, and a patient and a carrier with the *MECP2* duplication.[2] All the subjects provided written informed consent for the use of their phenotypic and genetic data. The proband's carrier mother, the patient and the carrier with the *MECP2* duplication are represented by a cross, triangle and square, respectively, in the control column. Data show the average values for fold differences relative to a normal male. Black bars represent mean values of each group.

The result of FISH suggests that *ATRX* duplication and *MECP2* duplication were occurred simultaneously resulting in complex genomic rearrangement. The proximal breakpoint of dup(X)(q21.1) and distal breakpoint of dup(X)(q28) were located on segmental duplications (Figure 1e) and the duplicated sequence at Xq21.1 existed near dup(X)(q28) (Figure 1f). 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.[14] Previous reports suggested that complex genomic rearrangements at Xq28 such as an embedded triplicated segment and stretches of non-duplicated sequence within dup(X)(q28) were probably mediated by FoSTeS,[7,15] and a particular genomic architecture, especially low copy repeats at distal breakpoints of dup(X)(q28), may render the *MECP2* region unstable. Thus, the dup(X)(q28) and dup(X)(q21.1) detected in our patients might be generated simultaneously by FoSTeS or other mechanism in a segmental duplication-dependent manner, suggesting the structural analysis of the entire X chromosome in patients with dup(X)(q28) to be important for understanding their correct clinical condition and providing appropriate education.

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APPENDIX

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Parental age and assisted reproductive technology in autism spectrum disorders, attention deficit hyperactivity disorder, and Tourette syndrome in a Japanese population

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ABSTRACT

We investigated whether advanced parental age and assisted reproductive technology (ART) are risk factors in autism spectrum disorders (ASDs), attention deficit hyperactivity disorder (ADHD), and Tourette syndrome (TS). Clinical charts of Japanese outpatients with ASD ($n = 552$), ADHD ($n = 87$), and TS ($n = 123$) were reviewed. Parental age of individuals with ASD, ADHD, or TS was compared with parental age in the general population (GP) of Tokyo after adjusting for year of birth. Paternal and maternal ages were significantly higher in persons with ASD and ADHD, but not those with TS. In final steps of stepwise logistic regression analysis, both maternal and paternal age were associated with ASD after controlling for the other parent's age, gender, and birth order. In cases where the presence or absence of ART could be ascertained (ASD $n = 467$; ADHD $n = 64$; TS $n = 83$), the rate of ART in cases of persons with ASD (4.5%) was 1.8 times the frequency expected in the GP, while ART was not present in cases of persons with ADHD and TS. These preliminary results remain tentative pending replication with larger, community-based samples.

1. Introduction

The etiology of autism spectrum disorders (ASDs) is not well understood. Recent studies suggest that *de novo* mutations (Marshall et al., 2008; O'Roak et al., 2011; Pinto et al., 2010; Sebat et al., 2007) and epimutations (Grafodatskaya, Chung, Szatmari, & Weksberg, 2010) in the genome play a role in ASD. Advanced parental age at delivery and assisted reproductive technology (ART) may be mediating factors in this process (Bonduelle et al., 2002; Manipalviratn, DeCherney, & Segars, 2009; Rivera et al., 2008). An association between advanced parental age and ASD has been reported mainly in studies from the United

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States (Croen, Najjar, Fireman, & Grether, 2007; Durkin et al., 2008; Grether, Anderson, Croen, Smith, & Windham, 2009; King, Fountain, Dakhlallah, & Bearman, 2009; Shelton, Tancredi, & Hertz-Picciotto, 2010), Europe (Hultman, Sandin, Levine, Lichtenstein, & Reichenberg, in press), and Israel (Reichenberg et al., 2006). Many studies (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; Hultman et al., in press; King et al., 2009; Reichenberg et al., 2006; Shelton et al., 2010) have reported similar significant associations between the occurrence of ASD and advanced parental age. However, several questions remain.

The first question is whether advanced parental age directly affects the development of ASD symptoms (i.e., difficulties with social situations and/or reciprocal communication with people), or whether it affects intellectual or general aspects of child development that could be associated with the risk of an ASD. A previous study reported an association between advanced paternal age and lowered intelligence quotient (IQ) in the general population (Saha et al., 2009), but it also found an association between advanced maternal age and higher IQ. Most previous studies have not separately investigated parental age and ASD according to whether or not the child displayed some degree of mental retardation (MR). One exception may be Tsuchiya et al. (2008), who identified advanced paternal age in individuals with ASD and IQ > 70, but the use of a control group unmatched for sex and age means that caution must be used when interpreting results: mean parental age in cases where ASD was present was five years less than mean parental age in the control group. These results could be affected by the fact that parental age in Japan has increased over the last few decades. Thus, the issue remains to be clarified.

If advanced parental age affects general brain development, it is reasonable to hypothesize that this impact might also be observed in other developmental disorders, such as attention deficit hyperactivity disorder (ADHD). Thus far, however, only one study has investigated this issue in relation to ADHD, and it found no association with parental age (Gabis, Raz, & Kesner-Baruch, 2010).

The second question is whether the association of advanced parental age with ASD is confined to cases of advanced paternal age, advanced maternal age, or both. Two recent large-scale population-based studies conducted in Europe and Israel reported an association between ASD and paternal age, but not maternal age (Hultman et al., in press; Reichenberg et al., 2006). In the United States, one study reported an association with maternal age (King et al., 2009), while four studies reported associations with both paternal and maternal ages (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; Shelton et al., 2010). Thus, the issue remains to be clarified.

In Asian populations, in contrast to Western populations, few studies have investigated the association between ASD and parental age. Case–control studies in Japan and China found an association with paternal age (Tsuchiya et al., 2008; Zhang et al., 2010). Another study (Koyama, Miyake, & Kurita, 2007) found that both paternal and maternal ages were elevated in cases of persons with ASD, compared with place-matched general population data.

The present study also attempted to explore whether an association exists between assisted reproductive technology (ART) and ASD. ART includes in vitro fertilization with trans-cervical embryo transfer (IVF) and intracytoplasmic sperm injection (ICSI). The number of children born using ART has been sharply increasing in the past decades in Japan and other developed countries. The first IVF child was reported in the UK in 1978 (Steptoe & Edwards, 1978). The first ICSI child was born in Belgium in 1992 (Palermo, Joris, Devroey, & Van Steirteghem, 1992). In Japan, the first IVF child was reported in 1983, and the first ICSI child in 1994 (Yanagida et al., 1994); in 2006, 1.8% of all Japanese newborns were by ART (Japan Society of Obstetrics and Gynecology, 2008).

ART may have the potential to affect the genetic and/or epigenetic structure of the genome in gametes, fertilized eggs, and embryos through its procedures and unnatural selection of germ cells. However, a limited number of studies have explored the impact of ART on developmental disorders, including ASD. Using birth registry data from Finland, Klemetti, Sevon, Gissler, and Hemminki (2006) compared cases of 4500 children born via IVF and 27,000 born via natural conception (NC), and found that ART was associated with a broad range of psychiatric conditions, including disorders of psychological, behavioral and emotional development. Knoester et al. (2007) followed children born after ICSI, IVF, and NC. After diagnosing these children at the age of 5–8 years based on parental report, they identified ASDs in three of 87 children born via ICSI; none in 85 children born via IVF; and one in the 81 children born via NC. These results may be inconclusive due to the small sample size.

Using the Danish national registry (Maimburg & Vaeth, 2007) compared the frequency of assisted conception (AC) in persons with autism and age-, sex- and birth place-matched healthy controls. The frequency of AC was not higher in autism (2.3%; $n = 461$) than in controls (5.4%; $n = 461$). Another study using the Danish registry found the incidence of ASD to be 0.68% in children born via AC ($n = 33{,}000$) and 0.61% in children born via NC ($n = 556{,}000$) (Hvidtjorn et al., 2010). The effect of AC was not found to be significant after adjusting for confounders. These studies did not separately analyze ART and other AC (ovulation induction and/or intrauterine insemination). Thus, there are few studies of the impact of ART on developmental disorders, including ASD, and those that do exist tend to be inadequate.

In an attempt to explore these questions, we conducted a chart review study of persons diagnosed with ASD and other developmental disorders, including ADHD and Tourette syndrome (TS). Parental age and frequency of ART were investigated for each of these three disorders.

2. Methods

2.1. Setting and procedures

We conducted a retrospective chart review of persons diagnosed with ASD, ADHD, and TS who first visited the child psychiatry outpatient clinic of the University of Tokyo Hospital, from April 2006 to March 2009. These disorders were the

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three most frequent disorders at the outpatient clinic during the period. Diagnosis was made according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria (APA, 2000) by child psychiatrists. Many patients were referred from rehabilitation facilities for children or private clinics.

Most (>90%) of the patients lived in the Tokyo Metropolis area, which has a population of approximately 13 million people. Generally, 70–90% of the cost for the medical care is covered by public health insurance in Japan.

Our review of clinical charts allowed the identification of 814 persons diagnosed with ASD, ADHD, or TS. We subsequently excluded cases of persons born before 1975 ($n = 33$), those who were not Japanese ($n = 10$), or those with nervous or systemic diseases ($n = 4$; three persons in the ASD group (postoperative brain tumor, postencephalopathy, and neurofibromatosis type 1) and one in the ADHD group (Turner Syndrome)). Five pairs of co-affected twins (four cases of monozygotic twins and one case of dizygotic twins) were found in the ASD group (there were none in either the ADHD or TS groups). For these cases, the co-twin with the more severe degree of disorder was included for each twin pair. This resulted in a pool of 552 persons diagnosed with ASD, 87 with ADHD, and 123 with TS. Of these individuals, we were able to determine whether or not ART had been used in 467 of the cases of ASD, 64 of the cases of ADHD, and 83 of the cases of TS.

2.2. Data analysis

Clinical charts were reviewed by two of the authors and the demographic and clinical information was studied, including sex, birth year, ages of mother and father at the delivery, education levels of the parents, use/no use of ART for the individuals birth, concurrence of MR, singleton/multiple birth, birth weight and gestational age at the delivery.

The information of ART was obtained from a questionnaire filled in by the parents at the visits of the clinic and from the clinical record written by the attending doctors or psychologists. IQ was measured using a Japanese version of Wechsler test (WISC-III ($n = 313$), WAIS-III ($n = 68$) or WPPSI ($n = 3$), according to the age of the subjects) or a Japanese version of Binet test (Tanaka–Binet test, $n = 98$).

This study was approved by the Ethics Committee of the Faculty of Medicine at the University of Tokyo.

General population (GP) data of Tokyo Metropolis was employed as control data for the parental ages and other variables (Ministry of Health, Labour and Welfare 2008). Average maternal age of the GP in the birth year was used as the "control maternal age" for each subject. Average of the control maternal age was then calculated in each disorder (ASD, ADHD and TS). Average of the control paternal age was calculated by the same method. The maternal and paternal ages in each disorder was compared with the control ages using one-sample t-test.

In ASD, stepwise logistic regression analysis was conducted to estimate the effect of parental age after controlling the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order as potential confounding factors. The GP data of Tokyo in 2002 was used as the control, because the mode birth year in ASD subjects was 2002.

All statistical analyses were conducted using SPSS version 17.0 (SPSS, Chicago, IL, US).

3. Results

3.1. Demographic features

We analyzed a total of 762 persons with either ASD ($n = 552$), ADHD ($n = 87$), or TS ($n = 123$). In the 552 ASD cases, 205 (37%) were diagnosed with autistic disorder (163 males and 42 females), and the rest were diagnosed with Asperger disorder or Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) ($n = 347$; 264 males and 83 females). Age at the time of first visit to the clinic was 9.8 ± 6.6 in the ASD group, 15.4 ± 6.3 in the ADHD group, and 11.4 ± 6.4 in TS group (mean $\pm$ SD; years). The mode (range) of birth years was 2002 (1975–2006) in the ASD group, 2001 (1975–2003) in the ADHD group, and 1996 (1975–2003) in the TS group.

Demographic features of the individuals with these three disorders are summarized in Table 1. For reference, population data for all children born in 2002 in Japan (2002 is the mode birth year for the ASD group). No significant difference was found in either paternal or maternal educational level among the three disorders.

3.2. Parental age

Table 2 summarizes mean parental ages at delivery in persons studied and in the general population of Tokyo. The mean ages in general population data were adjusted for the distribution of birth years in the individuals studied. Compared with the general population of Tokyo, mean paternal age and maternal age were significantly higher in persons with ASD (34.2 ± 5.8 and 31.6 ± 4.7 years, respectively) and ADHD (35.1 ± 6.2 and 32.2 ± 4.9 years, respectively). No difference was observed in persons with TS.

Among persons with ASD, paternal and maternal ages were significantly higher in those with autistic disorder than those with Asperger disorder or PDD-NOS (35.2 ± 6.1 vs. 33.6 ± 5.5 years for paternal age, $t = 2.58$, df = 368, $p = 0.01$; and 32.3 ± 4.5 vs. 31.2 ± 4.7 years for maternal age; $t = 2.19$, df = 381, $p = 0.03$, t-test, respectively). Maternal age was significantly higher in persons with autistic disorder and Asperger disorder or PDD-NOS than in the general population of Tokyo. While paternal age was significantly higher in persons with autistic disorder than in the general population of Tokyo, no difference was observed for persons with Asperger disorder or PDD-NOS.

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Table 1
Characteristics of Japanese patients with ASD, ADHD, and TS, and general population data of Tokyo in 2002.

	ASD		ADHD		TS		General population data of Tokyo in 2002	
	No.	%	No.	%	No.	%	No.	%
Gender								
Male	427	77%	74	85%	82	67%	51,619	52%
Female	125	23%	13	15%	41	33%	48,499	48%
IQ ≤ 70	146	32%	1	1%	5	14%	NA	
IQ > 70	310	68%	69	99%	31	86%	NA	
Singleton	534	97%	84	97%	123	100%	97,843	98%
Multiple birth	18	3%	3	3%	0	0%	2275	2%
Gestational age, weeks								
<37	36	7%	3	4%	5	5%	62,289	5%
≥37	472	93%	72	96%	101	95%	1,091,074	95%
Birth weight for gestational age								
<2500 g	51	10%	5	6%	1	1%	9162	9%
≥2500 g	480	90%	75	94%	115	99%	90,935	91%
Birth order								
1st	360	67%	56	70%	78	65%	53,947	54%
(Single child only)	(168)	31%	(29)	36%	(31)	26%	NA	
2nd	137	26%	20	25%	34	28%	34,594	35%
≥3rd	37	7%	4	5%	8	7%	11,577	12%
Paternal education								
Less than high school	17	3%	4	5%	4	3%	NA	
High school graduate	113	22%	19	25%	34	29%	NA	
Some college	323	64%	50	65%	70	60%	NA	
≥4-year college graduate (postgraduate)	55	11%	4	5%	8	7%	NA	
Maternal education								
Less than high school	11	2%	2	3%	4	3%	NA	
High school graduate	150	29%	26	33%	44	38%	NA	
Some college	345	67%	52	65%	69	59%	NA	
≥4-year college graduate (postgraduate)	12	2%	0	0%	0	0%	NA	
Paternal age at delivery, years								
<30	66	18%	6	13%	24	37%	471,069	40%
30–34	150	41%	21	47%	27	42%	401,772	34%
35–39	104	28%	7	16%	10	15%	216,070	18%
≥40	50	14%	11	24%	4	6%	86,088	7%
Maternal age at delivery, years								
<30	133	36%	11	24%	33	51%	685,322	58%
30–34	157	42%	18	40%	25	38%	371,265	32%
35–39	73	20%	16	36%	6	9%	105,838	9%
≥40	20	5%	1	2%	2	3%	12,574	1%

Abbreviations: ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; TS, Tourette syndrome; IQ, intelligence quotient; NA, not available.

No difference in paternal and maternal age was observed between males and females with ASD.

We also examined differences in parental age in persons with ASD and with/without MR. No significant difference was observed in either paternal or maternal age in persons with ASD and with/without MR. There was no significant correlation between full IQ and either paternal or maternal age ($r = -0.07$, $p = 0.25$, and $r = -0.06$, $p = 0.32$, respectively, Spearman correlation). Parental ages in persons with ASD and with/without MR were significantly higher than those in the general population of Tokyo.

We conducted stepwise logistic regression analysis in the ASD group using 2002 population data for Tokyo as a control, in order to test the effect of parental age after adjusting for the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order as potential confounding factors (Table 3). In the final steps of stepwise method, three variables were selected: the other parent's age, gender, and birth order. The adjusted ORs (95% confidence interval) for the ASD group were 1.8 (1.3–2.4), 2.1 (1.4–3.0), and 2.0 (1.3–3.1) in cases where fathers were aged 30–34, 35–39, and ≥40 years, respectively, when compared with those aged <30. Regarding maternal age, the adjusted OR was 2.9 (1.7–5.1) in cases where mothers were aged ≥40 years compared with those <30, but there was no significance when maternal age was under 40. In contrast, the results for birth order suggested that the decline in ASD risk associated with increasing birth order.

3.3. Assisted reproductive technology (ART)

Information about the use of ART was available for 467 persons diagnosed with ASD, 64 with ADHD, and 83 with TS born after 1988. No person identified was born using ART before 1989. ART was used in 21 cases where the person was

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Table 2
Mean paternal and maternal ages at delivery in persons with ASD, ADHD, and TS, and general population data in Tokyo after adjustment for birth year.

	Paternal age at birth, years ± SD		Maternal age at birth, years ± SD	
		General population data in Tokyo, after adjustment for birth year		General population data in Tokyo, after adjustment for birth year
ASD	34.2 ± 5.8 (n = 370)	33.0	31.6 ± 4.7 (n = 383)	30.4
ADHD	35.1 ± 6.2 (n = 45)	32.8	32.2 ± 4.9 (n = 46)	30.2
TS	31.9 ± 5.0 (n = 65)	32.7	29.8 ± 4.5 (n = 66)	29.6
Autistic disorder	35.2 ± 6.1 (n = 135)	33.1	32.3 ± 4.5 (n = 139)	30.5
ASP or PDD-NOS	33.6 ± 5.5 (n = 235)	33.0	31.2 ± 4.7 (n = 244)	30.3
Male ASD	34.0 ± 5.4 (n = 276)	NA	31.6 ± 4.6 (n = 285)	NA
Female ASD	34.8 ± 6.8 (n = 94)	NA	31.7 ± 4.9 (n = 98)	NA
ASD with MR	34.6 ± 6.2 (n = 78)	33.0	31.8 ± 4.4 (n = 80)	30.3
ASD without MR	33.8 ± 5.7 (n = 208)	32.9	31.4 ± 4.9 (n = 215)	30.3

Abbreviations: ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; TS, Tourette syndrome; ASP, Asperger disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; MR, mental retardation; SD, standard deviation; NA, not available.

Table 3
Distribution of ASD group and general population data of Tokyo in 2002 by parental age categories and other independent risk factors selected at final steps of stepwise logistic regression analysis, and unadjusted and adjusted odds ratios with 95% confidence intervals.

	ASD group		General population data of Tokyo in 2002		Unadjusted OR	95% CI		Adjusted OR[a]	95% CI		p value
	No.	%	No.	%							
Paternal age at delivery, years											
<30	60	17%	28,160	29%	1.0	Reference		1.0	Reference		
30–34	138	40%	36,921	38%	1.8	1.3	2.4	1.8	1.3	2.6	<0.001
35–39	98	28%	22,754	23%	2.0	1.5	2.8	2.1	1.4	3.0	<0.001
≥40	49	14%	10,270	10%	2.2	1.5	3.3	2.0	1.3	3.1	0.003
Maternal age at delivery, years											
<30	113	33%	40,845	42%	1.0	Reference		1.0	Reference		
30–34	143	41%	39,536	40%	1.3	1.0	1.7	1.1	0.9	1.5	0.369
35–39	70	20%	15,617	16%	1.6	1.2	2.2	1.4	1.0	2.0	0.058
≥40	19	6%	2107	2%	3.3	2.0	5.3	2.9	1.7	5.0	<0.001
Gender											
Male	256	74%	50,600	52%	2.7	2.1	3.4	2.7	2.1	3.5	<0.001
Female	89	26%	47,505	48%	1.0	Reference		1.0	Reference		
Birth order											
1st	229	66%	52,879	54%	1.0	Reference		1.0	Reference		
2nd	89	26%	34,246	35%	0.6	0.5	0.8	0.5	0.4	0.7	<0.001
≥3rd	27	8%	10,980	11%	0.6	0.4	0.8	0.4	0.3	0.6	<0.001

The variables as potential confounding factors, the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order, were submitted to a stepwise logistic regression model.
Abbreviations: ASD, autism spectrum disorder; CI, confidence interval; OR, odds ratio.
[a] Adjusted for the other parent's age, gender, and birth order.

diagnosed with ASD, but was not used in cases where the person was diagnosed with ADHD or TS. The characteristics of the 21 persons born using ART are summarized in Table 4. The observed number (n = 21) was 1.8 times the expected number (n = 11.7), which was estimated according to population data in Tokyo (Japan Society of Obstetrics and Gynecology, personal communication, 2011), after adjusting for the distribution of birth years in cases studied.

4. Discussion

4.1. Parental age at delivery

We conducted a retrospective chart review to investigate parental age at delivery and the use of ART in persons diagnosed with ASD, ADHD and TS. Parental age was elevated in cases of ASD in our Japanese population. We also found advanced

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Table 4
Number and characteristics of births using assisted reproductive technology in persons with ASD born after 1988.

	ASD ($n = 467$)
Number of births via assisted reproductive technology	21 (4.5%)
Male/female	16/5
Birth year (mode (range))	2003 (1989–2006)
Multiple birth/singleton	9/11
Autistic disorder/ASP or PDD-NOS	13/8
ASD with MR/without MR	6/7
Paternal age, years ± SD	38.9 ± 4.2
Maternal age, years ± SD	37.8 ± 4.0

Abbreviations: ASD, autism spectrum disorder; ASP, Asperger disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; MR, mental retardation; SD, standard deviation.

parental age in cases of ADHD, but not in TS, compared with GP data matched for birth place and year. These results suggest that elevated parental age is associated on a significant level not only in ASD, but in some other child-onset disorders, such as ADHD, as well. It is not clear why the association was not significant in TS. The nature of disturbance in brain development might be different in TS compared with ASD and ADHD, or it could be due to methodological limitations of this study, including the method of case selection and sample size.

No parental age difference was observed in those persons with ASD and with/without MR. This suggests that the effect of parental age on the development of ASD may not be specifically through an impact on intellectual development.

This raises the question of whether parental age affects specific domains of ASD symptoms, such as disorders in social interaction, communication, and restricted patterns of interested behavior, or whether it affects the development of more general brain functions. We did not evaluate specific symptoms of ASD in the present study. However, advanced parental age was also observed in persons with ADHD, not only in those with ASD. In addition, several studies have observed advanced parental age in schizophrenia (Torrey et al., 2009), which is also considered to be related to early neurodevelopment disturbances (Rapoport, Addington, Frangou, & Psych, 2005). These findings indicate that the effect of parental age might be on general aspects of brain development, not on a specific brain function. This may in turn later affect several aspects of brain function related to social interaction and cognition. This speculation would be consistent with the absence of advanced parental age observed in persons with TS, as the symptom spectrum of TS may be more confined to specific disturbances such as tics, rather than ASD or ADHD (APA, 2000).

Whether paternal age, maternal age, or both have an effect on the development of ASD has been controversial in previous studies (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; King et al., 2009; Koyama et al., 2007; Reichenberg et al., 2006; Shelton et al., 2010; Tsuchiya et al., 2008; Zhang et al., 2010). We conducted logistic regression analysis in the group with ASD, with place-matched GP data as a control. Results supported the hypothesis that both paternal and maternal ages tend to be elevated in ASD, after adjusting for the age of the other parent (Table 3). The magnitude of the effect of paternal age (OR = 1.8–2.1 for fathers of age 30 years or over) might be comparable to results of previous studies that observed an association with ages of both parents ((Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009); OR = 1.2–1.4). Similar to these studies, the association with maternal age was significant when age was ≥40 years.

This study suggested that the risk of ASD was highest for firstborn children and declined with increasing birth order, similar to two previous studies (Croen et al., 2007; Durkin et al., 2008). A possible explanation for the birth order effect is that parents having a child with ASD may tend to rarely have subsequent children because of the demands of parenting a child with a disability or concerns about genetic susceptibility (Jones & Szatmari, 1988).

4.2. Assisted reproductive technology (ART)

We performed a preliminary study of the frequency of the three disorders (ASD, ADHD and TS) in cases where ART (IVF and ICSI) was used. The hypothesis behind this is that ART may be associated with the risk of developmental disorders, possibly through its impact on genomic and epigenomic structure. An increase of *de novo* microscopic chromosomal anomalies has been observed in children born via ICSI (Bonduelle et al., 2002). The incidence of Beckwith–Wiedemann syndrome and Angelman syndrome due to epigenetic anomalies was also greater in children born using ART (Manipalviratn et al., 2009). Few studies have explored the association between ART and child-onset developmental disorders.

In the present study, cases of ASD had a rate of ART use of 4.5% (21 out of 466 cases), which was 1.8 times the expected frequency, based on GP data for Tokyo (2.5%) (Japan Society of Obstetrics and Gynecology, personal communication, 2011). In contrast, no cases of ART use were found in persons diagnosed with ADHD or TS.

However, when only the singleton individuals were examined, no difference was observed in the frequency of ART use between persons with ASD and GP data of Tokyo. The rate of multiple births was significantly higher in ASD cases where ART was used compared to where it was not. Thus, our preliminary study did not provide evidence for an increased rate of ASD in cases where ART was used. These results should be interpreted carefully because parental age was significantly higher in ASD cases where ART was used compared to where it was not.

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4.3. Strengths and limitations

Individuals in the present study were all Japanese (at least both parents were Japanese), which means that the sample tended toward ethnic homogeneity. This may be a strength compared with studies in other populations.

Limitations must also be acknowledged. First, general population data of Tokyo, taken from national statistics, were used as control data in this study. We adjusted the data according to the respective distribution of birth years in persons with ASD, ADHD, and TS. However, results were not adjusted for the sex of children and their socioeconomic status, including the education level of the parents. This could affect the results.

In contrast, the education levels of parents did not appear to be significantly different in persons with any of the three disorders. Therefore, it may not have an impact on any comparison of the disorders. Second, the general population may include individuals with ASD, ADHD, and TS. The respective prevalence of ASD and ADHD in Japan has been reported as being 1.2% (Honda, Shimizu, & Rutter, 2005) and 7.7% (Kanbayashi, Nakata, Fujii, Kita, & Wada, 1994). Epidemiological studies of persons with TS have not been conducted in Japan, but in other countries the prevalence has been estimated at around 1% (Robertson, 2008). With regard to persons with ASD and TS, the impact of having similarly affected persons in the general population data might be small on the present results, considering their low prevalence. In persons with ADHD, however, it could have some impact results.

Third, data was obtained by reviewing clinical charts, and some information was missing. This decreased the number of the persons who could be included in the statistical analyses. Fourth, sample size was limited, especially in relation to ADHD and TS. Finally, diagnoses were not made using structured interviews/observations, such as the Autism Diagnosis Interview-Revised (ADI-R) (Le Couteur, Rutter, & Le Couteur, 2003) and the Autism Diagnosis Observation Schedule (ADOS) (Lord, Rutter, DiLavore, & Risi, 1999), because those diagnostic tools were not available in Japan during the study period. Although diagnoses were made by skilled child psychiatrists (those with >10 years of experience), this could also be a weakness of the present study.

In summary, the present study observed elevations in both paternal and maternal age in persons with ASD and ADHD, but not in those with TS. In the case of ASD, the effect of parental age might not be significantly different between persons with and those without MR. In a preliminarily analysis, the frequency of ART use appeared to be higher in cases of persons with ASD than in the general population.

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