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Two Patients With Atypical Interstitial Deletions of 8p23.1:
Mapping of Phenotypical Traits

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Chromosomal 8p23 deletion syndrome is recognized as a malformation syndrome with clinical symptoms of facial anomalies, microcephaly, mental retardation, and congenital heart defects. The responsible gene for the heart defects in this syndrome has been identified as *GATA4* on 8p23.1. Two patients with interstitial deletions of 8p23.1 were investigated; one patient showed moderate developmental delay and Ebstein anomaly, and the other showed mild delay and typical atrioventricular septum defect. The precise deletion sizes, 17 and 2.9 Mb, were determined by FISH analyses using BAC clones as probes. The latter deletion was the smallest deletion including *GATA4* in the previously reported patients, and the critical regions and genes for clinical manifestation of 8p23 deletion syndrome, including facial anomalies, microcephaly, behavioral abnormality, and developmental delay, were discussed. © 2008 Wiley-Liss, Inc.

Key words: chromosome 8; *GATA4*; congenital heart defect; deletion; atrial septal defect

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INTRODUCTION

From an accumulation of the results of genotype–phenotype correlation derived from patients with congenital heart defects the chromosome 8p23.1 region has been highlighted as the critical region for heart malformation [Marino et al., 1992; Digilio et al., 1993; Wu et al., 1996]. Detailed analyses narrowed the region, and indicated *GATA4*, a zinc finger transcription factor, as a candidate gene [Marino et al., 1992; Digilio et al., 1993; Bhatia et al., 1999; Devriendt et al., 1999; Pehlivan et al., 1999; Giglio et al., 2000]. Subsequently Garg et al. [2003] identified *GATA4* mutations in patients with congenital heart defects. A *GATA4* mutation completely segregated with atrial septal defect (ASD) in a large family, and many mutations have been reported to date [Okubo et al., 2004; Hirayama-Yamada et al., 2005]. Recently, important functions have been demonstrated in *Gata4* mice, that is, a general role of myocardial *Gata4* in regulating cardiomyocyte proliferation and a specific stage-dependent role in regulating the morphogenesis of the right ventricle and the atrioventricular canal [Zeisberg et al., 2005]. Gata4

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mice is also expressed in endothelial-derived cells in the development of heart valves [Rivera-Feliciano et al., 2006]. Thus, GATA4 is quite important in the development of the heart.

The chromosome 8p23.1 region is unique not only for its association with congenital heart malformations, but also for common polymorphic inversion mediated by two olfactory receptor gene-clusters within an interval of 4.7 Mb, which act as low copy repeats (LCRs) [Sugawara et al., 2003]. The olfactory receptor gene superfamily is the largest in the mammalian genome, and several of the human olfactory receptor genes appear in clusters with more than 10 members located on almost all human chromosomes [Giglio et al., 2001]. Sometimes such LCRs can cause chromosomal rearrangements, deletion and/or duplication, and they are recognized in various contiguous gene syndromes, for example Williams syndrome, 22q11.2 deletion syndrome, Smith–Magenis syndrome, and Sotos syndrome [Sugawara et al., 2003]. In these submicroscopic deletion syndromes, both ends of the deletion are on LCRs and the range of the deletion is common among the patients. However, there are some exceptional cases, in which the breakpoints of the chromosomal aberrations are larger or smaller than the common deletion between LCRs. Thus, it is very important to investigate the precise deletion region in atypical cases for further definition of the minimal critical deletion region in each phenotype.

Recently, we encountered two patients with 8p23 deletion syndrome, and detailed analyses of the deleted range showed atypical deletion size in both patients.

PATIENT REPORT

Patient 1

Patient 1 was a newborn baby boy, the second child of healthy parents. His father was 36 years old, and his mother, 33 years old. The mother had a history of spontaneous abortion at gestational age of 20 weeks. At 21 weeks gestation, his mother was referred to our hospital because of a tentative diagnosis of hypoplastic right heart and severe pulmonary artery atresia seen in her fetus by fetal echography. Oligohydramnios was also identified. At 37 weeks of gestation, he was born by induced delivery, with a weight of 2,400 g (<10 centile). He showed restriction in the bilateral hip joints with limitation in the range of movement. We noticed micrognathia as the only dysmorphic finding (Fig. 1A). A detailed echocardiographic examination revealed severe hypoplastic right heart, stenosis of the pulmonary artery, ASD, and mild regurgitation of the mitral valve due to stenosis (Fig. 1B). Oxygen supplementation was needed for 21 days after birth

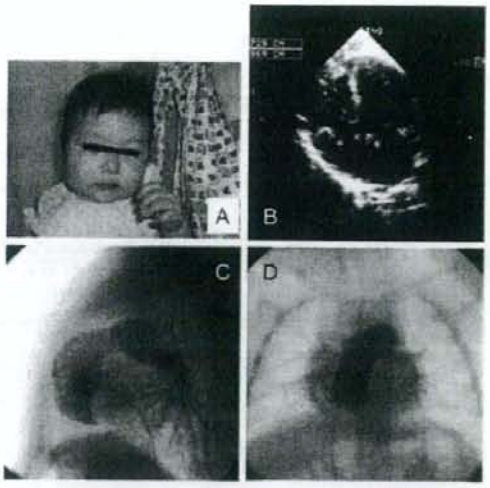

FIG. 1. Clinical examination of Patient 1. A photo taken when he was age 8 months shows only micrognathia as a dysmorphic finding (**A**). Echocardiography shows the diameter of the mitral valve and the tricuspid valve as 9.7 and 7.2 mm, respectively. This finding indicates hypoplasia of the right ventricles. Patent foramen ovale can be seen (**B**). Catheter examination revealed cardiac hypertrophy with a cardiothoracic ratio of 57%, and hypoplasia of the right heart with a right ventricular endodiastolic volume of 29.0 ml/m^2 (62% normal) (standard should be over 70% normal) (**C,D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

because of pulmonary artery hypertension. When he was 10 months old, he showed growth retardation with a weight of 7 kg (<3 centile), a length of 67.8 cm (<10 centile), and a head circumference of 41.8 cm (<3 centile). He could turn over from the supine to the prone position, but he could not sit up by himself; showing psychomotor delay. Finally, catheterization confirmed the diagnosis of Ebstein anomaly at this time (Fig. 1C,D).

Patient 2

Patient 2, a 14-year-old boy, was born at 38 weeks of gestation, with a birth weight of 2,900 g and a height of 49 cm. When he was 2 years old, a heart murmur was found, and subsequently detailed echocardiographic examination revealed an ASD (type II), small ventricular septal defect (VSD), pulmonary stenosis, and dilatation of the right ventricle. He also presented with gonadotropin-dependent early expression of puberty, thus anti-gonadotropin releasing hormone treatment was initiated at 7 years and 6 months. At age 13 years, he showed standard physical growth with a height of 151.6 cm (<50 centile) and a weight of 47.6 kg (<90 centile), but showed mild microcephaly with a head circumference of 52 cm (<50 centile). His facial appearance indicated prominent maxillary central incisors and mild micrognathia. He began walking at age 11 months and spoke his first words at age 2 years and 6 months. He had a febrile seizure at

age 3 years and 11 months. At that time, he already fed himself and had bladder control. He was very hyperactive during infancy but adapted well after his entrance into an elementary school for handicapped children. He showed moderate mental retardation with an IQ of 45 evaluated by Tanaka–Binet at age of 11 years. At age 14 years, he received education at junior high school for handicapped children and could calculate simple sum and read and writes simple sentences containing Chinese characters. Now surgical repairs are planned because of right ventricle volume overload.

The clinical findings of both patients together with the reported patients are summarized in Table I.

MATERIALS AND METHODS

DNA Extraction

Genomic DNAs were obtained from the peripheral blood of both patients and the parents of Patient 1 by the standard method.

Karyotype Analysis and Fluorescence In Situ Hybridization (FISH) Analysis

G-banded chromosome analysis and two-color FISH analysis were performed on metaphase chromosomes of the patients. Human genomic BAC clones were used as probes for FISH analysis,

TABLE I. Summary of Clinical Manifestations in Reported Patients and the Present Patients

	Terminal deletion																				
ID		#12	#2	#7	#1	#2	#1	#3	#4	#5	#3	#4	#5	#1	#2	#3	#3	#5	#1		
Reference	Digilio et al., 1993	/Giglio et al., 2000	Digilio et al., 1998		Pecile et al., 1990		Hutchinson et al., 1992				Digilio et al., 1998			Wu et al., 1996			Johnson et al., 1997		Devriendt et al., 1999	Bhatia et al., 1999	Gilmore et al., 2001
Karyotype	del (8) (p21)		del (8) (p21)	del (8) (p21)	del (8) (p22)	del (8) (p23.1)	del (8) (p23)	del (8) (p23)	del (8) (p23)	del (8) (p23)	del (8) (p23)	del (8) (p23)	del (8) (p23)	del (8) (p23.1)	del (8) (p23.1)	del (8) (p23.1)	del (8) (p23.1)	del (8) (p23.1)	del (8) (p21.3)	del (8) (p23.1)	del (8) (p23.1)
Growth delay																					
Low birth weight	+		+	+	−	+	−	+	−	−	+	−	−	−	+	+			+		−
Slow growth			+	−	−	+	−	−	+		−	−	−	−	−	+					−
Microcephaly	+		+	+	−	+	−	+	−	−	+	−	+		+				+		
Dysmorphology																					
Facial anomalies	+		+	+	+	+	+	+	+	+	+	+	+	−	+	+				+	−
High forehead					+		+	−	+	+											
Epicanthal folds	+		+	+	+	−	−	−	−	−	+	+	+								
Arched palate			+	−	+						+	+	+								
Micrognathia					+	+	+	+	+	+						+					
Low-set ears	+		+	+	+	+	+	+	+	+	+	+	+			+					
Malformed ears	+		+	+	+	−	+	+	+	+	+	+	+								
Widdy-spaced nipples	+		+	+	+	+	+	+	+	−	+	+	+			+					
Puffy hands and/or feet					+	+	+	+	+	−						+					
Gut malrotation																					
Diaphragmatic hernia						+															
Partial agenesis corpus callosum																					
Abnormal genitalia	+		+	+	−	−	−	+	+	−	+	+	−	−	−	+			+		
Cryptorchidism			−	+							−	−	−								
Hypospadias	+		+	+							+	+	−						+		
Early expression of puberty																					
Developmental disorder																					
Mental retardation (severe or mild)	+		+	+	±		+	+	+	+	+	+	+	+	+		+	+	+	D	−
Behavioral disturbance							+	+	+	−				+	+						
Delays in the acquisition of language																					+
Heart defect	+		+	+	+	+	−	+	−	+	+	+	+	−	+	+	+	+	+	+	−
Atrial septal defect (ASD)											+	+			+			+	+		
Ventricular septal defect (VSD)								+			+							+	+		
Atrioventricular canal defect	+		+	+		+							+				+				
Patent foramen ovale																					
Ebstein's anomaly								+													
Tetralogy of Fallot									+								+				
Pulmonary valve stenosis	+		+			+							+		+				+	+	
Persistent left superior vena cava											+									+	
Hypoplastic right ventricle	+		+																		
Others				+				+			+	+			+	+				+	

	Interstitial deletion																						
ID		#11		#1	#8	#2	#3	#4	#5	#6	#7	#8	#1	#2	#3	#4	#5	#2	#8	#3			
Reference	Marino et al., 1992	/Giglio et al., 2000	Moorman et al., 1992	Digilio et al., 1998		Devriendt et al., 1999	(/Claeys et al., 1997 #5)	(/Claeys et al., 1997 #3)			(/Claeys et al., 1997 #4)	(/Claeys et al., 1997 #2)	Pehlivan et al., 1999					Hutchinson et al., 1992	/Giglio et al., 2000	Slavotinek et al., 2005	Shimokawa et al., 2005	Patient 1	Patient 2
Karyotype	del (8) (p21.1p23.1)		del (8) (p21.3p23.1)	del (8) (p21p23)	del (8) (p21p23)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p22.1p23.1)	del (8) (p23.1p23.2)	del (8) (p23.1p23.1)	del (8) (p23.1p23.1)	del (8) (p23.1p23.2)	del (8) (p23.1p23.1)	del (8) (p22p23.1)		del (8) (p22p23.1)	del (8) (p23.1p23.1)	del (8) (p22p23.3)	del (8) (p23.1p23.1)
Growth delay																							
Low birth weight	+		−	+	−	+	+	+	+	+	−	+	+	−	−			−		−	+	+	−
Slow growth	+		+	+	+								−	+	−	+	+	+				+	−
Microcephaly	+		+	+	+	+	+	+	+	+	−	+	−	+	−		+	−		−	+	+	+
Dysmorphology																					−		
Facial anomalies	+		+	+	+			+	+		−	+	+	+	+			+		+	−	+	−
High forehead	+		+															−				−	−
Epicanthal folds	+		+	+	+			+				+						−				−	−
Arched palate	−		+	−	−								+		+		+	+			−	−	−
Micrognathia	+													+	+			+		+		+	−
Low-set ears	−		+	+	+							+	+					−			−	−	−
Malformed ears	+		+	+	+				+			+						+				−	−
Widdy-spaced nipples	−		+	−	−								+					+				−	−
Puffy hands and/or feet			±									+						−				−	−
Gut malrotation						+														+		−	−
Diaphragmatic hernia																				+	+	−	−
Partial agenesis corpus callosum												H						+				−	−
Abnormal genitalia	+			+	+	+												+				−	−
Cryptorchidism	+			+	−	+					+									+	+	−	−
Hypospadias	+			+	+																	−	−
Early expression of puberty																		+					+
Developmental disorder																							
Mental retardation (severe or mild)	+		+	+	+	+	+	+	+	+	+	+	+	+	±	+	±	+		D	D	+	+
Behavioral disturbance					−	+	+		+	+	+							−					+
Delays in the acquisition of language														+	+								+
Heart defect	+		−	+	+	+	+	+	+	+	−	−	+	+	+	+	−	+		+	+	+	+
Atrial septal defect (ASD)						+	+	+	+	+			+	+		+				+	+	+	+
Ventricular septal defect (VSD)						+		+						+						+			+
Atrioventricular canal defect	+			+	+										+								
Patent foramen ovale																		+				+	
Ebstein's anomaly																						+	
Tetralogy of Fallot															+								
Pulmonary valve stenosis	+			+		+	+	+	+	+			+			+		+				+	+
Persistent left superior vena cava	+			+	+																		
Hypoplastic right ventricle	+			+																		+	
Others	+			+	+			+					+										

H, hypoplasia of the corpus callosum; D, died in futal or early neonatal period.

as described elsewhere [Shimokawa et al., 2004]. Analysis for Patient 1 was carried out in the laboratory of Kyushu Medical Science in Nagasaki, using the RPCI 11.c BAC clone library (Invitrogen, Carlsbad, CA). Because Patient 2 was managed by the out patient clinic of Tokyo Women's Medical University Hospital, he was examined in the laboratory of the university mainly using the original human BAC clones, which were used for the Human Genome Project, supplied from the department of molecular genetics of Keio University as collaboration [Nusbaum et al., 2006].

Linkage Analysis Using STS Markers

Since only the parents samples from Patient 1 were obtained, linkage analysis of the family of Patient 1 was performed to determine the origin of the deletion, using the microsatellite markers, D8S277, D8S552, D8S518, and D8S264. Information about the primers of markers was obtained from the in-silico library (http://genome.ucsc.edu/). PCR amplification was performed according to the standard method. The amplicons were visualized by ethidium bromide staining after separation by electrophoresis on acrylamide gel.

Direct Sequencing

Nucleotide sequencing analysis of *GATA4* was performed in both patients to screen nucleotide alteration in the remaining allele by the direct-sequencing method, using big-dye terminator following the protocol of the manufacturer (AppliedBiosystems, Foster City, CA). The primers and conditions are described elsewhere [Hirayama-Yamada et al., 2005].

RESULTS

G-banded chromosomal analysis showed interstitial deletion of 8p in both patients (Fig. 2A). According to the detailed FISH analysis using BAC clones as probes, a large interstitial deletion over both LCRs with the size of 17 Mb was identified in Patient 1, and a smaller deletion than the LCRs interval with the size of 2.9 Mb including *GATA4* was determined in Patient 2 (Fig. 2B,D). The results of the FISH analyses are summarized in Table II.

Only the result of D8S277 located on 8p23.1 was informative for detection of the allelic origin of the deletion, and Patient 1 showed only one band which was the same as one of his father's bands (Fig. 2C). This indicates that the allele derived from his mother was deleted.

Sequencing analysis of *GATA4* showed no mutation in either patient.

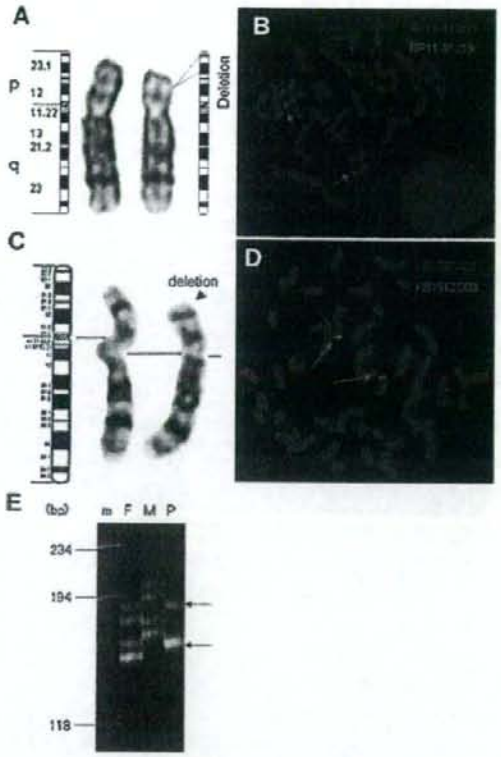

FIG. 2. Molecular and cytogenetic examinations. G-banded chromosomal analysis of Patients 1 and 2 shows interstitial deletion of 8p23, respectively (**A**,**C**). Two-color FISH analysis of Patient 1 using BAC clones labeled with spectrum orange (RP11-412I11) and spectrum green (RP11-91J19) indicates deletion of the orange signal (**B**). FISH analysis of Patient 2 using BAC clones labeled with spectrum orange (KB1597A01) and spectrum green (KB1512DO3) indicates deletion of the red signal (**D**). Electrophoresis of the PCR amplicons of D8S277 shows deletion of the maternally derived allele (**E**). Abbreviations m, F, M, P indicates molecular size marker, father, mother, and Patient 1, respectively. Patient 1 shows only one band which is the same as one of her mother's bands. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

With a broad clinical spectrum from normal intelligence to severe mental impairment and from minor malformations to fatal abnormalities, the characterization of 8p23 deletion syndrome has been elusive [Gilmore et al., 2001; Giglio et al., 2002; Slavotinek et al., 2005]. Reported extracardiac findings common to several patients include postnatal growth retardation, microcephaly, facial anomalies including micrognathia and low set ears, mental retardation, behavioral disturbance, genitourinary anomalies, and congenital diaphragmatic hernia [Pecile et al., 1990; Hutchinson et al., 1992; Morrison

TABLE II. Summary of FISH Analysis

Clone name	Band*	Start*	End*	FISH result Patient 1	FISH result Patient 2	Remarks
KB1512D03	8p23.3	158,644	337,693		+/+	
RP11-91J19	8p23.3	304,160	465,319	+/+		
RP11-43A14	8p23.3	600,704	801,642	+/+		
RP11-784G19	8p23.3	969,278	1,165,773	+/+		
RP11-412I11	8p23.3	1,790,660	1,962,749	+/−		
RP11-121F7	8p23.2	3,284,324	3,324,954	+/−		> MYOM2-REPs
RP11-3N20	8p23.2	4,817,346	4,991,651	+/−		
KB1043B11	8p23.2	4,988,199	5,138,926		+/+	
RP11-143D15	8p23.2-p23.1	6,182,456	6,365,021	+/−		
KB1521E03	8p23.1	6,915,190	7,178,018		+/+	
KB1015E10	8p23.1	7,653,826	7,654,978		+/+	< Contig gap (LCR)
RP11-399J23	8p23.1	8,156,720	8,327,336	+/−		
KB1585B11	8p23.1	8,196,622	8,348,477		+/+	
KB1588A04	8p23.1	8,699,363	8,850,259		+/+	
KB1551B10	8p23.1	9,063,688	9,231,856		+/+	
KB1089E11	8p23.1	9,223,546	9,224,546		+/+	
KB1071H04	8p23.1	9,333,815	9,334,895		+/+	
KB1591G07	8p23.1	9,483,788	9,647,131		+/−	
KB1160H06	8p23.1	9,863,649	10,031,483		+/−	
KB1597A01	8p23.1	11,511,508	11,686,119		+/−	
RP11-118D21	8p23.1	11,597,791	11,778,975	+/−		
KB811B3	8p23.1	11,599,162	11,654,918		+/−	> *GATA4* coverage
RP11-589N15	8p23.1	11,627,389	11,803,111	+/−		
KB1772D08	8p23.1	11,714,501	11,881,439		+/−	
KB1170H02	8p23.1	12,202,030	12,277,184		+/+	< Contig gap (LCR)
KB1076A04	8p23.1	12,424,949	12,425,882		+/+	
KB1013B10	8p23.1	12,476,782	12,478,039		+/+	
KB1156E01	8p23.1	12,415,364	12,610,203		+/+	
RP11-252C15	8p23.1-p22	12,586,955	12,762,845	+/−		
KB1509C11	8p23.1	12,736,303	12,897,159		+/+	
KB1587G04	8p22	13,243,112	13,395,129		+/+	
RP11-100K3	8p22	13,877,522	14,041,348	+/−		
KB1565C07	8p22	13,952,770	14,099,525		+/+	
RP11-23H1	8p22	15,027,287	15,191,603	+/−		
RP11-274K12	8p22	16,009,761	16,162,045	+/−		
RP11-950E13	8p22	18,191,583	18,388,324	+/+		
RP11-593B12	8p21.3	19,056,836	19,236,416	+/+		
RP11-204M16	8p21.2	23,257,419	23,431,673	+/+		

*Chromosomal band and location on UCSC database (May 2004).
Gray cells indicate deletion areas.

et al., 1992; Wu et al., 1996; Johnson et al., 1997; Devriendt et al., 1999; Pehlivan et al., 1999; Giglio et al., 2000; Gilmore et al., 2001; Shimokawa et al., 2005; Slavotinek et al., 2005]. Our patients shared some of these common findings including growth retardation, developmental delay, microcephaly, facial anomalies, and cardiac malformation (Table I).

There are many reported patients with terminal telomeric deletion of chromosome 8 short arm. However, there is much confusion since many reports published in 20th century were reported as terminal deletions. But, later the same patients were re-evaluated by advanced molecular and cytogenetic techniques including microsatellite markers analyses and FISH analyses, and were reported again as interstitial deletion not terminal deletion. The modification of nomenclature of karyotyping also caused confusion. We readjusted the reported patients and classified them into two groups, patients with terminal deletion and those with interstitial deletion (Table II). There are at least 19 reported patients who showed interstitial deletion of 8p23.1 region [Hutchinson et al., 1992; Marino et al., 1992; Morrison et al., 1992; Claeys et al., 1997; Digilio et al., 1998; Devriendt et al., 1999; Pehlivan et al., 1999; Giglio et al., 2000; Shimokawa et al., 2005; Slavotinek et al., 2005] (Table I). In this group, many patients shared a common deletion, del(8)(p23.1p23.1). In the in-silico database of human genome sequence, there are two gaps in contig in the 8p23.1 region (Table II). These two gaps work as LCRs and result in polymorphic inversion and also interstitial 8p23.1 deletion syndrome as described above. Five cases reported by Devriendt et al. [1999] (#2–6) and the patient reported by Shimokawa et al. [2005] were investigated not only by karyotyping but also by advanced methods such as microsatellite marker

analyses and FISH analyses, and determined to be sharing common deletion region.

The results of Patient 1 showed a larger deletion than the common deletion mediated by these LCRs [Shimokawa et al., 2004, 2005]. The size was about 17 Mb, and both ends of the deletion were beyond LCRs (Table II). Recently, new LCRs lying at 8p23.3, MYOM2-REPs, were reported to be related to a new rearrangement, whereas the breakpoint of the deletion of this patient was far beyond these new LCRs [Giorda et al., 2007] (Table II).

On the other hand, Patient 2 showed a smaller range with a size of 2.9 Mb, and the proximal end of the deletion was at the LCR, but the telomeric end was at the center between two LCRs (Table I, Fig. 3). Devriendt et al. [1999] reported a male patient (#7) who showed a smaller deletion of less than 2 Mb, but not including *GATA4* region (Fig. 3). He showed a normal heart and a normal appearance, but showed mild mental retardation and severe behavioral problems. The telomeric end of the deletion of Patient 2 reported in this manuscript may overlap with that of the patient (#7) reported by Devriendt et al. (Fig. 3). Thus, the critical region for mild mental retardation and behavioral problems would be narrowed to this region. In this region, there is only one gene, *TNKS*, which is suspected to be related to the telomere function and highly expressed in the brain [Smith et al., 1998]. On the other hand, the critical region for microcephaly and mild facial dysmorphism, which is not manifested in the patient (#7) reported by Devriendt et al., but is seen in our Patient 2, was narrowed to the centromeric half region between two LCRs. In this region, at least 11 genes are located. *MSRA*, which is highly expressed in kidney and nervous tissue, carried out the enzymatic reduction of methionine sulfoxide to methionine, and its proposed function is the repair of oxidative damage to proteins to restore biological activity [Moskovitz et al., 1996]. *SOX7*, which encoded a member of the SOX family of transcription factors involved in the regulation of embryonic development and the determination of the cell fate [Takash et al., 2001]. It is well-known that mutations in *SOX3*, one of the SOX family, have been associated with X-linked mental retardation with growth hormone deficiency [Laumonnier et al., 2002]. Thus, these genes might be related to the patients' microcephaly and dysmorphisms.

Patient 2 also showed precocious puberty, and there is another reported patient with del(8)(p23) and early expression of puberty [Hutchinson et al., 1992]. However, this symptom can often be seen in congenital anomaly syndromes, and is not specific to some chromosomal regions. Hence, we cannot determine that this co-occurrence is specific or coincidential.

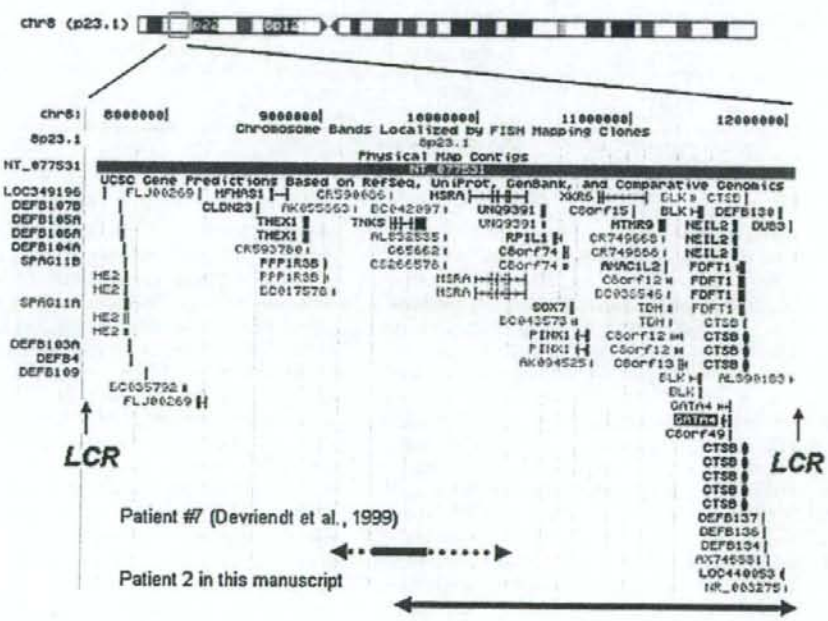

FIG. 3. Physical map of common deletion region of 8p23.1. Thin horizontal bars with arrowheads on both sides indicate deletion region, and dotted bars indicate ambiguity.

GATA4 was included in the deleted region in both patients. Congenital heart defects in both patients can be explained by haploinsufficiency of *GATA4*. Patient 2 showed typical findings with ASD and VSD. However, Patient 1 showed Ebstein anomaly, which is quite rare in this syndrome, and only one such patient has been reported to date [Hutchinson et al., 1992]. Thus, there might be some other genetic modifications in Patient 1. One of the hypotheses would be loss of heterozygosity of *GATA4*. To deny it, we analyzed the sequences of the homologous allele of *GATA4* in the remaining normal chromosome 8 in both patients, but identified no mutation. Since a previously reported patient with Ebstein anomaly also showed a large deletion karyotyped as del(8)(p23), this condition might be derived from other deleted gene(s) in the 8p deletion. More information about genotype–phenotype correlation in patients with 8p23 deletion syndrome should be accumulated.

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De-novo balanced translocation between 7q31 and 10p14 in a girl with central precocious puberty, moderate mental retardation, and severe speech impairment

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No causative gene has been found for idiopathic central precocious puberty; and *FOXP2*, located in 7q31, is the only known gene for speech and language disturbances. We report a girl with central precocious puberty, moderate mental retardation, and severe speech impairment; accompanied by a de-novo balanced translocation between 7q31 and 10p14. Physical mapping through molecular cytogenetic investigations demonstrated the breakpoints of 7q31 and 10p14 within a bacterial artificial chromosome (BAC) clone RP11-124G5 and a cosmid clone derived from a BAC clone RP11-1122C18, respectively. *FOXP2* was found to be localized approximately 500 kb distant from the centromeric end of the disrupted BAC RP11-124G5 at the 7q31 breakpoint. Speech impairment in the girl might be derived from dysfunction of *FOXP2* by a position effect of the 7q31 translocation breakpoint. *Clin Dysmorphol* 17:31–34 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: central precocious puberty, de-novo balanced translocation, *FOXP2*, position effect, speech impairment

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Introduction

Whereas the great majority of cases of de-novo balanced translocations are clinically normal, some result in congenital malformations (Warburton, 1991). Phenotypic abnormalities have been explained by various mechanisms: direct disruption of a gene; position effect; uniparental disomy; and cryptic deletion or duplication (Kumar *et al.*, 1998). The association of two or more cases with a Mendelian disorder and balanced translocations with one breakpoint in common, are strongly of a causative gene localized at the breakpoint. It is difficult, however, to confirm whether the gene disruption is causal for the disorder or coincidental, when only one case is reported to have the breakpoint or the condition is extremely rare (Kondoh *et al.*, 2002).

Here, we report a girl with central precocious puberty, moderate mental retardation, and severe speech impairment; accompanied by a de-novo balanced translocation between 7q31 and 10p14. To date, no causative gene has been found for idiopathic central precocious puberty; and *FOXP2* (forkhead box P2), localized at 7q31, is the only known gene for speech and language disturbances (Lai *et al.*, 2001). We mapped the translocation breakpoints through molecular cytogenetic analyses, to investigate the possibility that a gene (s) for central precocious puberty, mental retardation, and speech impairment in this girl was (were) disrupted at the breakpoints.

Clinical report

The girl, born to healthy nonconsanguineous parents, had moderate retardation of gross motor development (head control at 5 months and walking alone at 21 months) and mental development (IQ 40 at the age of 6 years). She showed growth acceleration from the age of 5 years, and exhibited accelerated pubertal development with breast development at the age of 7 years, pubic hair at the age of 8 years, and menarche at the age of 8 years. When seen by us at the age of 8 years, she weighed 35.5 kg (± 2.0 SD) and height 144 cm (± 3.2 SD). She had a depressed nasal root, a bulbous nose, thick lips, and a high arched palate; narrow shoulders, large hands (Fig. 1), and a left inguinal hernia, though she did not have any recognizable

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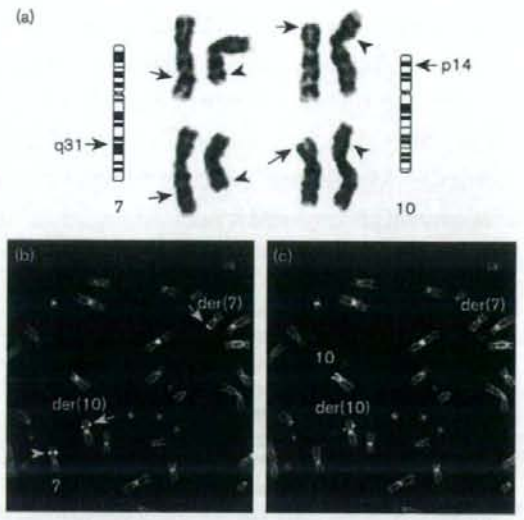
Fig. 1

Patient at the age of 8 years.

congenital malformation syndrome. Tanner pubertal stage (Tanner, 1962) was 4 in breast development and 3 in pubic hair. Bone age was 11 years, according to the modified Tanner–Whitehouse 2 (TW-2) method (Murata, 1993). Serum follicle-stimulating hormone was 8.8 mIU/ml, luteinizing hormone 7.1 mIU/ml, and estradiol (E2) 33.8 pg/ml at the baseline. After stimulation by gonadotropin-releasing hormone, serum follicle-stimulating hormone and luteinizing hormone increased to 19 and 58 mIU/ml, respectively. These findings fulfilled the criteria for central precocious puberty (Tanaka *et al.*, 2005). Brain MRI was normal. G-banded chromosomes of lymphoblastoid cell lines showed a balanced translocation 46,XX,t(7;10)(q31;p14) (Fig. 2a). The parents had normal chromosomes. Treatment with buserelin acetate, a gonadotropin-releasing hormone analogue, was followed by cessation of premature growth spurt, pubertal development, and menstruation. When last seen by us at age

Fig. 2



(a) G-banded partial karyotype. The breakpoints on derivative chromosomes 7 [der(7)] and 10 [der(10)] are indicated by arrowheads, and those on normal chromosome 7 and 10 by arrows. (b) FISH analysis using the RP11-124G5 probe. Three signals are observed: one on normal chromosome 7 (arrowhead), and one each on the der(7) and der(10) (arrows). (c) FISH analysis using the RP11-1122C18 probe on the same metaphase as (b). Three signals are observed: one on normal chromosome 10 (white arrowhead), and one each on the der(7) and der(10) (white arrows). FISH, fluorescence in-situ hybridization.

18 years, she showed normal development of breast and pubic hair, and her menstruation cycle was normal. She had been unable to speak a word, expressing herself with gestures; whereas she had normal hearing and moderate deficiency of language comprehension, and was independent in daily activities.

Methods

To map the translation breakpoints at 7q31 and 10p14, we performed fluorescence in-situ hybridization (FISH) analysis on metaphase chromosomes prepared from lymphoblastoid cell lines of the girl. We used 11 bacterial artificial chromosome (BAC) clones around the 7q31 breakpoint (RP11-80L6 [7q22.1], RP11-12L9, RP11-92J13, RP11-415F5 [7q31.1], RP11-78C11 [7q31.1-q31.2], RP11-103A1, RP11-124G5, RP11-140G6, RP11-95L16 [7q31.2], Rp11-105B19 [7q31.31], RP11-66F23 [7q32.1]), and 16 BAC clones around 10p14 breakpoint (RP11-67B16 [10p15.1], RP11-379F12, RP11-164D13, RP11-358M19, RP11-481K2, RP11-939G16, RP11-1112K8, RP11-1122C18, RP11-585D24, RP11-381D12, RP11-48A13, RP11-187J14, RP11-138I18, RP11-1076H20, RP11-347I22 [10p14], RP11-124N14 [10p12.33]), according to the Genome Browser website

maintained by the Genome Center at University of California Santa Cruz (Kent *et al.*, 2002). These BAC clones were labeled with SpectrumGreen-11-dUTP or SpectrumOrange-11-dUTP (Vysis, Downers Grove, Illinois, USA) using the nick-translation method, and hybridized to metaphase chromosomes.

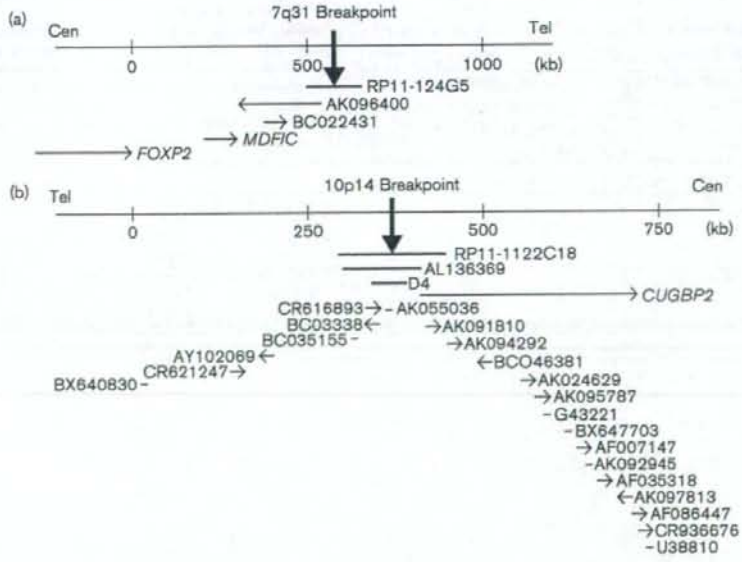
Results and discussion

The BAC clones RP11-124G5 and RP11-1122C18 were found to be disrupted by the breakpoints of 7q31 and 10p14, respectively (Fig. 2b and c). For further mapping of 10p14 breakpoint; where abundant messenger RNAs (mRNAs) are assigned in GenBank (Benson *et al.*, 2004), according to the Genome Browser website (Kent *et al.*, 2002); we performed FISH analysis using cosmid clones derived from RP11-1122C18. To construct a cosmid contig from the BAC clone; purified BAC DNA, partially digested with *Sau*3AI, was ligated to Super-Cos1 cosmid vector according to the manufacturer's protocol (Stratagene, La Jolla, California, USA), followed by PCR-based sequence tagged site content mapping (Kamimura *et al.*, 2004). A cosmid clone, named as D4, was found to be disrupted by the breakpoint (data not shown).

Physical mapping of 7q31 breakpoint is shown in Fig. 3a. Within the disrupted clone RP11-124G5 (approximately 170 kb), we found only an mRNA AK096400. We found a gene *MDFIC* (MyoD family inhibitor domain containing isoform) and an mRNA BC022431 in a 300-kb region to the centromere, and no gene or mRNA in a 300-kb region to the telomere. *MDFIC*; expressed in lymphoid organs, prostate, uterus, and small intestine; encodes a protein involved in transcriptional regulation of viral genome expression (Thebault *et al.*, 2000; Young *et al.*, 2003). *FOXP2*, approximately 500-kb distant from the centromeric end of the disrupted BAC RP11-124G5, encodes a transcription factor expressed in developing brain. A missense mutation, a nonsense mutation, and a direct disruption of the gene were found in patients with developmental speech and language deficits (Lai *et al.*, 2001; MacDermot *et al.*, 2005). Thus, dysfunction of *FOXP2* by a position effect of 7q31 translocation breakpoint was assumed to be a possible molecular etiology for speech impairment in the girl, considering that a breakpoint could disturb a gene function from 3 to 900-kb region, and both from upstream and downstream of the gene (Kleinjan and van Heyningen, 1998). Analysis of chromatin structure around the breakpoint and/or functional study of cultured cells would give more concrete evidences in the pathogenesis.

Physical mapping of 10p14 breakpoint is shown in Fig. 3b. Within the disrupted clone RP11-1122C18 (approximately 155 kb), we found a gene *CUGBP2* (CUG triplet repeat, RNA binding protein 2) and six mRNAs. We also found, outside RP11-1122C18, 12 mRNAs in a 300-kb

Fig. 3



Physical map of the genomic region around the 7q31 (a) and 10p14 (b) breakponits. Arrows represent directions of transcription.

region to the centromere and three mRNAs in a 300-kb region to the telomere. The disrupted cosmid clone D4, located approximately between 40 and 80-kb region from the telomeric end of a sequenced genomic DNA AL136369, encompasses whole coding regions of two mRNAs (CR616893 and AK055036), and a part of an mRNA BC033338. AK055036 and CR616893, expressed in brain, could be candidate genes for mental retardation in the girl; whereas BC033338, expressed in testis, is not likely to be a candidate. *CUGBP2*; expressed in muscular systems, central nervous system, thymus, digestive tract, lung, and kidney; has been implicated in the regulation of nuclear and cytoplasmic RNA processing events including alternative splicing, RNA editing, stability, and translation (Ladd and Cooper, 2004). The gene is assumed to be a candidate for thymus hypoplasia/aplasia in partial monosomy 10p (Lichtner *et al.*, 2002). It is unclear whether dysfunction of the gene could cause some features of the girl.

In a recent array, comparative genomic hybridization-based study by Gribble *et al.* (2005), six of 10 patients with *de novo* apparently balanced translocations, phenotypic abnormalities, and mental retardation had submicroscopic structural chromosomal imbalances close to, or distant from, the translocation breakpoints. Not a position effect but a cryptic deletion of *FOXP2* might cause speech impairment in the girl. A deletion of *FOXP2* could not be excluded, because no BAC clone encompassing the gene was used in FISH analyses in this study. It is also possible that her features and de-novo balanced translocation were coincidental events.

In conclusion, we report a girl with central precocious puberty, moderate mental retardation, and severe speech impairment; accompanied by a de-novo balanced translocation between 7q31 and 10p14. Speech impairment in this girl might result from dysfunction of *FOXP2*, by a position effect of 7q31 breakpoint. Molecular etiologies of mental retardation and central precocious puberty in this girl remain unknown.

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Clinical Report

Craniosynostosis in a Patient With a de novo 15q15-q22 Deletion

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Interstitial deletions involving the chromosomal band 15q15 are very rare. A total of five cases were previously reported. Here another case of a 15q15.2-q22.2 deletion is reported, presenting with severe craniosynostosis of coronary, metopic, and sagittal sutures. The chromosome 15 with the 17.7-Mb deletion was of the paternal origin. A critical region for craniosynostosis may be located at the 734-kb segment at 15q15.2. Interestingly, the entire *FBN1* gene was deleted in this patient. © 2008 Wiley-Liss, Inc.

Key words: 15q interstitial deletion; craniosynostosis; *FBN1* deletion

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INTRODUCTION

Constitutional deletions involving the chromosomal band 15q15 are very rare. Only five patients have ever been reported: a 15q13-q15 deletion [Autio et al., 1988], a 15q15.2-q21.2 deletion [Koivisto et al., 1999], and three similar deletions of 15q15-q22.1 [Fukushima et al., 1990; Hutchinson et al., 2003; Shur et al., 2003]. Interestingly, craniosynostosis was noted in two cases [Fukushima et al., 1990; Shur et al., 2003]. Here, we report on a girl with severe craniosynostosis and a 15q15.2-q22.2 deletion. The deletion was precisely mapped by FISH analysis for the first time. A critical region for craniosynostosis will be discussed.

CLINICAL REPORT

The patient, a girl, was born at 41 weeks of gestation as a second child to a 33-year-old mother and a 39-year-old father, both healthy and nonconsanguineous. Birth weight was 2,760 g (−1.5 SD), length 47 cm (−0.9 SD), and OFC 29.5 cm (−2.0 SD). Turricephaly, facial dysmorphism, and abnormal toes were noted. Intermittent positive pressure ventilation was started 30 min after birth. Right tension pneumothrax with pneumoperitoneum and subcutaneous emphysema was treated for 10 days by tube-drainage. Recurrent vomiting due to pylorostenosis was relieved by pyloromyotomy at age 1 month. She was tube-fed. Craniosynostosis of the coronary, metopic, and sagittal sutures was diagnosed with plain radiography at age 2 months. Increased intracranial pressure was relieved with

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cranial vault distraction at age 9 months (3D-CT image of the cranium right before the operation is shown in Fig. 1D). When referred to us at age 13 months, she was hypotonic, and showed profound growth and psychomotor retardation. She could not control her head or fix her gaze. Her facial abnormalities included thin and bow-shaped eyebrows, downslanting palpebral fissures, mild blepharophimosis; low-set ears with stenosis of bilateral ear canals; a nose with bulbous nasal tip, hypoplastic alae nasi and upturned nostrils; high arched palate, bifid uvula, epiglottis hypoplasia; a thin upper lip and tucked-in lower lip (Fig. 1A,B). She had a short neck with low occipital hair line. Mild pectus excavatum and accessory nipples were seen. She had small, edematous hands and feet, long fingers, adducted and proximally placed thumbs, clinodactyly of the fifth fingers, four finger creases, overlapping 3rd and 5th over 4th toes, and right sandal foot with metatarsus adductus (Fig. 1C). At age 18 months, her weight was 6,700 g (−3.3 SD), length 72.4 cm (−2.5 SD) and OFC 38.4 cm (−5.4 SD). Ophthalmologic examination indicated strabismus, occasional horizontal nystagmus, and optic nerve atrophy. Waking electroencephalography showed pronounced basic wave abnormalities including continuous and diffuse high-voltage fast waves dominant in the occipital area and intermittent, and synchronized rhythmic slow waves in the bilateral parietal area. Brain magnetic resonance imaging did not reveal any abnormalities. Ultrasonographic examination showed no abnormalities of the heart or visceral organs. Now aged 19 months, she is tube-fed, is unable to raise her head or smile socially.

MOLECULAR CYTOGENETIC ANALYSIS

G-banded chromosomes of peripheral blood lymphocytes from the proband had a karyotype was 46,XX,del(15)(q15.2q22.1) (Fig. 1E). FISH analysis was done using BAC clones mapped to 15q15-q23 (Fig. 1F) showed a 17.7-Mb deletion spanning BAC RP11-2o16 to BAC RP11-945a12 [UCSC genome browser coordinate (version Mar. 2006): chr 15: 41,149,653–58,889,686 bp]. Parental chromosomes were normal. The chromosome 15 with the deletion was of paternal origin, as proven with a SNP marker (rs4328375: father C/C, mother G/G, and the patient G/-).

DISCUSSION

The 19-month-old girl with 15q15.2-q22.2 deletion we described had a severe craniosynostosis in addition to multiple minor abnormalities. Craniosynostosis has been described in two unrelated patients with 15q15-q22.1 deletions [Fukushima et al., 1990; Shur et al., 2003]. Band 15q15 is likely to be critical for craniosynostosis, in view of the fact that patients with deletions of 15q21 (three patients), 15q21-q22 (four patients), 15q21-q24 (one patient), and q22-q25 (one patient) were without craniosynostosis [Fryns et al., 1982; Yip et al., 1987; Formiga et al., 1988; Martin et al., 1990; Liehr et al., 2003; Pramparo et al., 2005; Ades et al., 2006; Lalani et al., 2006].

We compared the deletion in the girl we described with deletions in three other patients in the literature [Hutchinson et al., 2003; Pramparo et al., 2005] (Fig. 1F). None of the three patients had craniosynostosis. Deletions in two patients [Pramparo et al., 2005] were analyzed with array CGH. The patient described by Hutchinson et al. [2003] had a 15q-q22.1 deletion including *MFAP1* and *FBN1*, proven by analysis with FISH clones. It was deduced that the critical region for craniosynostosis is a 734-kb segment (UCSC genome browser coordinates Mar. 2006: chr 15: 41,149,653-41,884,025 bp) between RP11-2o16, non-deleted in the girl we described, and *MFAP1*, deleted in the patient described by Hutchinson et al. [2003] but without craniosynostosis. The segment contains at least 22 known genes, of which *UBR1*, *EBP42*, and *STRC* may be excluded as the candidates for craniosynostosis, because recessive mutations of these genes are responsible for Johanson-Blizzard syndrome [Zenker et al., 2005], anemias [Rybicki et al., 1988] and non-syndromic deafness [Verpy et al., 2001].

A cluster of oppositely imprinted genes are known to be present in the 15q11-q13 region, deletions of which result in Prader–Willi or Angelman syndrome. Shur et al. [2003] postulated that deletion of the putative gene for craniosynostosis in paternally derived chromosome 15 results in mild phenotype, and that in maternally derived chromosome 15 results in severe one. However, this is not likely to be the case in view of the fact that in the patient we described the deleted chromosome 15 was paternally derived, yet her phenotype was severe.

FBN1 at 15q21, a gene responsible for Marfan syndrome, was deleted in the patient we described. Haploinsufficiency of *FBN1* is likely to contribute at least in part to Marfan syndrome phenotype [Hutchinson et al., 2003; Judge et al., 2004; Matyas et al., 2007]. The patient we described is 19 months old, does not show any sign of the syndrome. This may be due to her young age. Two patients with complete deletion of *FBN1* showed marfanoid habitus [Hutchinson et al., 2003; Ades et al., 2006]. Their ages are 10 and 42 years.

FBN1 mutations are usually unrelated to craniosynososis as Marfan syndrome presents with no craniosynostosis. However, at least two cases of Shprintzen–Goldberg syndrome (SGD; having craniosynostosis) with *FBN1* mutations have been reported [Mizuguchi and Matsumoto, 2007]. This may imply that other genetic factors in addition to *FBN1*

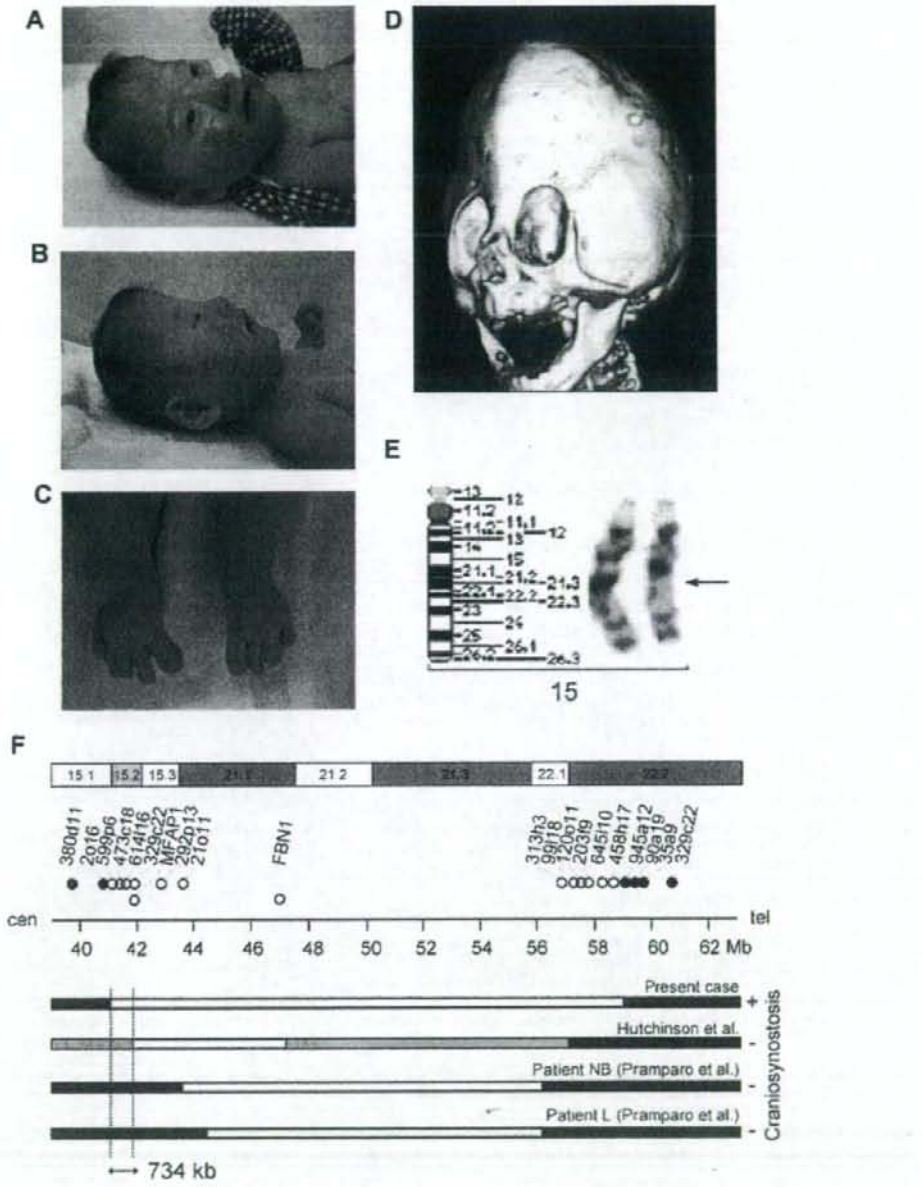

FIG. 1. **A–C**: Photographs of the patient at age 19 months. Deformed head after cranioplasty, tubular nose with hypoplastic alae nasi, and thin upper lip were recognized. Right sandal foot with metatarsus adductus and bilateral overlapping 3rd, 4th, and 5th toes were noted. **D**: 3D-CT image of cranium at age 9 months before cranioplasty, demonstrating bilateral coronary, metopic, and sagittal synostosis. **E**: Partial karyotype of the patient. Arrow indicates deletion. **F**: Schematic presentation of the 15q15-q22 deletion in the present patient, Patient Del(15) [Hutchinson et al., 2003], and Patients NB and L [Pramparo et al., 2005]. Chromosomal bands for 15q15.1-q22.2 are shown at the top. Above the genomic scale, position of BAC/cosmid clones was indicated as circle. Black and white circles are intact and deleted clones in the patient, respectively. Thick black, white and gray horizontal lines indicate intact, deleted and untested regions of the cases whose deletion sizes are available at the molecular cytogenetic level. Minimal critical region (734 kb) for the craniosynostosis, from RP11-2o16 to *MFAP1*, is presented as bidirectional arrow.

aberrations in the two SGD cases might be important for craniosynostosis. In this context, deletion of a candidate gene in the 734-kb critical region and *FBN1* may contribute to the severe craniosynostosis in the present case.

The patient with 15q15-q22 deletion we described had right sandal foot with metatarsus adductus and bilateral overlapping of the 3rd and 5th toes over 4th toe. These features are similar to those of Shur's patient [Shur et al., 2003] who showed metatarsus adductus and overlapping toes, and Pramparo's patient [Pramparo et al., 2005] with five overlapping toes. These patients had deletions at 15q15-q22.1, or 15q21.1-q22.1. Thus a gene(s) for these foot manifestations is likely to be at 15q21.1-q22.1.

In conclusion, 15q15.2-q22.2 deletion was analyzed at the precise molecular cytogenetic level for the first time. The deletion was 17.7 Mb in size and of the paternal origin. A 734-kb critical region at 15q15.2 for craniosynostosis could be proposed through the mapping of the deletion.

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Short Report

Alu-related 5q35 microdeletions in Sotos syndrome

Mochizuki J, Saitsu H, Mizuguchi T, Nishimura A, Visser R, Kurotaki N, Miyake N, Unno N, Matsumoto N. *Alu*-related 5q35 microdeletions in Sotos syndrome.
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Haploinsufficiency of the *NSD1* gene due to 5q35 microdeletions or intragenic mutations causes Sotos syndrome (SoS). In 46 of the 49 Japanese deletion cases, common deletion breakpoints were located at two flanking low copy repeats (LCRs), implying that non-allelic homologous recombination (NAHR) between LCRs is the major mechanism for the common deletion. In the other three cases of atypical deletions, the mechanism(s) of deletions remains unanswered. We characterized the atypical microdeletions using fluorescence *in situ* hybridization (FISH), quantitative real-time polymerase chain reaction (qPCR), and Southern blot hybridization. All the deletion breakpoints in the three cases were successfully determined at the nucleotide level. Two deletions are 1.07 Mb (SoS19) and 1.23 Mb (SoS109) in size, and another consisted of two deletions with sizes of 28 kb and 0.72 Mb, intervened by an intact 29-kb segment (SoS44). All deletions were smaller than a typical 1.9-Mb common deletion. *Alu* elements were identified in both deletion breakpoints in SoS19, one of deletion breakpoints in SoS109, and both deletion breakpoints of the proximal 28-kb deletion in SoS44. *Alu*-mediated NAHR is strongly suggested at least in two of atypical deletions. Finally, qPCR is a very useful method to determine deletion breakpoints even in repeat-related regions.

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Key words: Sotos syndrome – microdeletion – *Alu* – non-allelic homologous recombination

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Alu elements are the member of short interspersed elements, occupying 10.6% of the human genome sequence with more than 1×10^6 copy numbers (1). Remarkable homology (especially for younger *Alu* families with a small number of random mutations as little as 0.1% pairwise divergence) (2), high density in the genome and active transposability would be associated with genomic instability. Thus, *Alu* elements could be substrates of non-allelic homologous recombination (NAHR) and may stimulate non-homologous end joining (NHEJ) especially for non-recurrent rearrangements (3). *Alu*-mediated NAHR may cause various diseases, including hypercholesterolemia

low density lipoprotein receptor (LDLR), alpha-thalassemia (alpha-globin), angioneurotic adema (C1 inhibitor), and breast cancer (BRCA1) [see a review by Deininger et al. (4)].

Sotos syndrome (SoS; OMIM 117550) is a congenital disorder characterized by childhood overgrowth, mental retardation and craniofacial dysmorphic features. Nuclear-receptor-binding SET domain-containing protein 1 (*NSD1*) gene at 5q35 was isolated as the causative for SoS (5). In European descents, intragenic *NSD1* point mutations are the major cause, while in Japanese microdeletions involving the whole *NSD1* gene are the most frequent. At the proximal and distal deletion breakpoints of the typical 1.9-Mb microdeletion, highly homologous sequences, low copy repeats (LCRs) were identified (6). Within these LCRs, major recombination hotspots of the common microdeletion have been determined (7). LCR-mediated NAHR could be the most common mechanism for SoS microdeletions.

We previously analyzed 112 SoS cases consisting of 95 Japanese and 17 non-Japanese cases. Of the 95 Japanese patients, 49 (52%) had a microdeletion, whereas only 1 (6%) of the non-Japanese case had a deletion. Among these 50 microdeletions, 46 had the common 1.9-Mb deletions whose breakpoints were located at the two LCRs (6). The remaining four cases (three Japanese and one non-Japanese) had smaller microdeletion with somehow different breakpoints.

Here, we analyzed the three Japanese cases of atypical deletions and could successfully characterize all the deletion breakpoints at the nucleotide level. Mechanisms for atypical deletions in SoS will be discussed.

Methods

Cases

Three Japanese SoS patients (SoS19, SoS109 and SoS44) with atypical microdeletions were analyzed. The diagnosis was made as previously described (6). Genomic DNA and cell pellets for fluorescence *in situ* hybridization (FISH) were prepared with standard methods from peripheral blood samples or lymphomatoid cell lines. Experimental protocols were approved by the Committee for Ethical Issues at Yokohama City University School of Medicine.

Quantitative real-time PCR

The microdeletion breakpoints were analyzed using genomic DNAs by quantitative real-time polymerase chain reaction (qPCR) with DyNAmo™ HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland) on Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia). A total of 21 primer pairs were designed using the online PRIMER 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as qPCR probes, including Q1 primer set (at 176,490 kb in chromosome 5, 4.7 kb proximal to *NSD1*), Q2 primer set (at 175,537 kb in chromosome 5) and Q3 primer set (at 177,343 kb in chromosome 5) (Table 1). Q3 primer set was assigned a relative concentration of 1.00 as a calibrator. The modified standard curve method (8) and the delta-delta C_t relative quantitative method were employed according to the manufacturer's protocol. Averages of duplicates were calculated by ROTOR-GENE 6000 SERIES software (Corbett Life Science, Sydney, Australia).

Southern hybridization

Breakpoint locations were characterized by Southern hybridization using *Eco*RI-, *Bgl*II-, and *Sph*I-digested SoS44 DNAs. Sufficient DNAs of SoS19 and SoS109 were not available for this analysis. Healthy female DNA was used as a normal control. A proximal probe (at 175,928 kb in chromosome 5) and a distal one (176,711 kb in chromosome 5) were labeled using DIG synthesis kit (Roche, Basel, Switzerland), hybridized, and washed according to the manufacturer's protocol. Images were captured on FluorChem (Alpha Inntech, San Leandro, CA). Primer information is available on request.

Fluorescence *in situ* hybridization

We performed FISH analysis on chromosomes of SoS44 lymphoblastoid cell line. Seven bacterial artificial chromosome (BAC) clones covering the common 5q35 deletion region (RP11-434l24, RP11-316c19, RP11-815i19, RP11-643a23, RP11-1008g19, RP11-99n22, and RP11-962j7) were selected as FISH probes, and their DNAs were labeled with FITC-dUTP or Cy5-dUTP (Invitrogen, Carlsbad, CA) by Nick translation kit (Vysis, Des Plaines, IL). Fluorescence photomicroscopy was performed under an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Direct sequencing

Fragments containing the deletion breakpoint were amplified by PCR for direct sequencing. Long PCR primers adjacent to presumed deleted

Table 1. Primers for quantitative real-time polymerase chain reaction

	Position (kb)	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
Common primer			
Q1	176490	TGAGAATCTATGGCGCTGTG	GGATGAACTGCTTGCTGACA
Q2	175537	TGCTACTGCTCAGGCTCTCA	TAAGGCACAGCAGCAAACAG
Q3	177343	ATTGTAATGCCCACACAGCA	GGGCTAGAAGTGGGAGAAGG
SoS19-specific primer			
q4	175721	GGGAGATGAGGTTGAGGACA	CGCACCTCTTAACCCTTCAT
q5	175757	CATTCCTGTTCCGTGACCTT	ATGCCTTAATGTGGGACTGG
q6	176802	CGGCCTCTGAGTAAGACTCG	GCAGAGGCAGACCTATCTGG
q7	176826	ATGGAGCTTACCAGCCACAC	TCAGTTCAGGAAGGGAGGTG
SoS109-specific primer			
q8	175851	GCAGCTGTATCATCCAACCA	ACCCTGGCAGATGTGGAGTA
q9	175859	CAGACCCAAGTGCTGAGACA	AAAGCCACACGTCCATTACC
q10	176978	GGGATCAGTTAGTTGGCTATGC	CGCGCTCCAGACTCTGAT
q11	176992	CATCTTGAGGTAGGCCCAGA	CCCACAGCAAGATGTGTCTC
q12	177057	TCTGCAGCTGAGGCAGAGT	CAGTGCCAGGCAGAGTCC
q13	177085	GTACTTTCCCAGCCCTCACA	GGGTGAAAGGAAATGGGATT
SoS44-specific primer			
q14	175931	CAGAACACCGCTTTCTCCAC	GGCTTTGGTTCTTGAAGCTG
q15	175933	CCAAAGAAGCCTCCAGCTCT	CAGCCTCTCCACCACCTAAG
q16	175944	GCGTGAATGTGAAAGACGTG	GCCTTGGTGCAGAGAATGTT
q17	176025	GGTGGCATTAAAGCAAGGAA	CAGGAAGGCCTCTTTGTGTC
q18	176699	AGCCCAGCCTAAGTGTGGTA	CACCGTGTGTGTTGACACCT
q19	176711	CTCACCTGGTGGTTCCAGAT	GTGGCCTGTTAGCACCGTC
q20	175966	CCTGCCTGTTTCCTTCTCTG	CCCAGGGAGAGGAGCTTATT
q21	175981	GAAGTGGGATGGAGGTGAAG	GGGAGCCTGAGGGTTATCTC

regions by qPCR were generated (available on request). Parental DNAs were available in all patients. PCR was cycled once at 94°C for 1 min, 35 times at 98°C for 10 sec, at 68°C for 5–10 min, and once at 72°C for 10 min in 25 μl mixture using LA-Taq (Takara Bio Inc., Otsu, Japan). PCR products were purified with ExoSAP™ (USB Co., Cleveland, OH) and sequenced using BigDye™ terminator (Applied Biosystems, Foster City, CA) on the ABI 3100 automatic DNA sequencer (Applied Biosystems). Deletion breakpoints were approached with a primer-walking manner (information of primers is available on request).

Sequence analysis

Sequences around breakpoints were analyzed using web sites including UCSC genome browser (http://genome.ucsc.edu/), RepeatMasker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) for screening of interspersed repeats and low complexity DNA sequences, BLAT (http://genome.brc.mcw.edu/cgi-bin/hgBlat) for finding DNA seqeuences of 95% and greater similarity of length 25 bases or more, and PRIMER 3 (http://frodo.wi.mit.edu/) to design primers for further analysis. DNASIS PRO software was also used (Hitachi Soft Engineering Co. Ltd, Tokyo, Japan) for general DNA analysis platform.

Results

Analysis of breakpoints

According to the standard curve method and/or the delta-delta C_t relative quantitative method, we could narrow down breakpoint locations. Heterozygous deletions were suggested in all patients from q5 to q6 (UCSC genome browser ver. Mar 2006: chromosome 5: 175,757–176,802 kb) in SoS 19, from q9 to q12 (chromosome 5:175,859–177,057 kb) in SoS109, and from q15 to q18 (chromosome 5: 175,933 kb-176,699 kb) in SoS44, respectively (Table 1, Fig. 1). It is interesting that there seems to be an island within the deletion in SoS44 as deleted points of qPCR were not continuous (Fig. 1c).

Long PCR amplification of segments containing breakpoints was performed, and 3.3 kb- and 1.1 kb-fragments in SoS19 and SoS109, respectively, could be successfully obtained, but not from their parents (Fig. 1a,b). However, PCR product in SoS44 could not be obtained, thus we performed Southern hybridization and FISH analysis to make sure that qPCR results of deletion(s) were correct and to obtain additional information. Proximal and distal probes at 175,928 and 176,711 kb in chromosome 5 were generated, adjacent to supposed deletion breakpoints. Aberrant bands were detected by the proximal probe in *Sph*I-digested DNA, but not in *Eco*RI- and *Bgl*II-digested DNAs (Fig. 1d). Using the distal probe,