Table 2 Mutation screening of candidate genes in 41 patients with Kabuki syndrome

Involving gene(s)	*Base substitution*	*Amino acid change*	*Patient(s) with KS*		*dbSNP*	*Allele frequency among unaffected Japanese*[a]	*Results of mutation screening*
			Homo	*Hetero*			
TRPM3	459C>T	A153A	0	1	NR	0	Synonymous
	4023G>A	S1341S	13	28	rs3739776	—	SNP, synonymous
KLF9	459C>T	V153V	0	1	NR	0	Synonymous
SMC5	916G>A	V306I	37	4	rs1180116	—	SNP, non-synonymous
	922T>C	C308R	21	10	rs1180117		SNP, non-synonymous
MAMDC2	62T>C	L21P	0	2	NR	0.02	SNP, non-synonymous
	492C>T	T164T	0	1	NR	0	synonymous
	816C>T	Y272Y	11	16	rs2296772	—	SNP, synonymous
	867G>A	A289A	13	15	rs2296773	—	SNP, synonymous
	1063_1065 delAAA	K355 del	11	17	rs61609258	—	(SNP) synonymous; del/ins polymorphism

Abbreviations: *KLF9*, Kruppel-like factor 9; *MAMDC2*, MAM domain containing 2; *SMC5*, structural maintenance of chromosomes protein 5; SNP, single nucleotide polymorphism; *TRPM3*, transient receptor potential cation channel, subfamily M, member 3; dbSNP, registration number of database of SNP (http://www.ncbi.nlm.nih.gov/SNP/).
[a]Allele frequency was calculated from 188 chromosomes of 94 individuals.

these genes are major genetic factors for KS. However, it is presumable that the genes have some etiological roles for KS because of its genetic heterogeneity. Ontology of the PANTHER classification suggested that the three genes were associated with developmental biology, such as mRNA transcription regulation. Moreover, the 1.27 Mb region of 9q21 was included in an earlier reported candidate locus of cleft lip/palate by meta-analysis of linkage analysis.[24] Patient K16 actually had velopharyngeal insufficiency because of a submucous cleft palate. Therefore, it is reasonable to consider that the deleted genes cooperated with the development of a cleft palate, which is often accompanied by KS.

Although the ~152 kb deletion within intron 5 of *C20orf133 (MACROD2)* in patient K6 did not involve any coding exon and her parents' DNAs were unavailable, the deletion was neither registered as CNV in DGV nor was it found in 95 normal Japanese individuals by qPCR (data not shown). Maas *et al.*[14] reported *de novo* ~250 kb deletion, including exon 5 of *C20orf133 (MACROD2)*, in a patient with KS. Direct sequencing for the gene in 62 other patients with KS did not detect mutations,[14,15] but the gene may be one of the causative genes for KS in consideration of its genetic heterogeneity.

We focused this study on KS on deletion/duplication detected using oligonucleotide array and mutation screening of the coding genes within the region. One limitation of this study is its resolution. As a matter of course, a higher resolution array can detect smaller genomic rearrangements, which were undetectable in the same patient, as we showed here compared with an earlier study of BAC array CGH.[18] Although SNP probes are useful to examine loss of heterozygosity as a collateral evidence in deletions, unevenly distributed probes of the SNP array have a disadvantage for CNV detection. As various platforms on oligonucleotide array have developed, higher resolution platforms will have to be applied to search tiny genomic rearrangements in patients with KS. Another limitation is that we assumed that a single copy number change caused KS. It remains to be elucidated whether CNV association[25] contributes towards manifestations of KS. If further investigation with refined array technologies cannot find the etiology of KS, the direction of study for KS will have to be changed to find *de novo* sequence alteration or methylation aberration, including in the non-coding genomic regions.

In summary, we applied molecular karyotyping with GeneChip 250K array to detect copy number aberrations in 17 patients with KS and screened four candidate genes in 41 patients with KS. We could not identify causative DNA alteration for KS, but the locus, 9q21.11-q21.12, including *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*, may contribute to the cleft palate of KS. Further investigations will be needed as various array platforms have the potential to specify genomic alterations for KS.

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Maternal Uniparental Disomy 14 Syndrome Demonstrates Prader-Willi Syndrome-Like Phenotype

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Objective To delineate the significance of maternal uniparental disomy 14 (upd(14)mat) and related disorders in patients with a Prader-Willi syndrome (PWS)–like phenotype.

Study design We examined 78 patients with PWS-like phenotype who lacked molecular defects for PWS. The *MEG3* methylation test followed by microsatellite polymorphism analysis of chromosome 14 was performed to detect upd(14)mat or other related abnormalities affecting the 14q32.2-imprinted region.

Results We identified 4 patients with upd(14)mat and 1 patient with an epimutation in the 14q32.2 imprinted region. Of the 4 patients with upd(14)mat, 3 had full upd(14)mat and 1 was mosaic.

Conclusions Upd(14)mat and epimutation of 14q32.2 represent clinically discernible phenotypes and should be designated "upd(14)mat syndrome." This syndrome demonstrates a PWS-like phenotype particularly during infancy. The *MEG3* methylation test can detect upd(14)mat syndrome defects and should therefore be performed for all undiagnosed infants with hypotonia. *(J Pediatr 2009;155:900-3).*

Maternal uniparental disomy 14 (upd(14)mat) is characterized by prenatal and postnatal growth retardation, neonatal hypotonia, small hands and feet, feeding difficulty, and precocious puberty.[1] Chromosome 14q32.2 contains several imprinted genes, and loss of expression of paternally expressed genes including *DLK1* and *RTL1* is believed to be responsible for upd(14)mat phenotype.[2] Thus far, 5 patients with epimutations and 4 patients with a microdeletion affecting the 14q32.2 imprinted region have been reported to have upd(14)mat-like phenotype.[2-4] Paternal uniparental disomy 14 (upd(14)pat) shows a distinct and much more severe phenotype characterized by facial abnormality, bell-shaped thorax and abdominal wall defects.[1] Initially, upd(14)mat was identified in patients with Robertsonian translocations involving chromosome 14, but increasing numbers of patients with a normal karyotype have been recognized.[1,5] Because maternal uniparental disomy 15 is responsible for the condition in more than 20% of patients with Prader-Willi syndrome (PWS), of which the overall prevalence is more than 1 in 15000 births,[6] one could suspect that upd(14)mat is underestimated. Phenotype of upd(14)mat is known to resemble that of PWS, which is characterized by neonatal hypotonia, small hands and feet, mental retardation, and hyperphagia resulting in obesity beyond infancy. Mitter et al[7] recently reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS and raised the question that upd(14)mat could be present in patients with PWS-like phenotype. Thus we examined patients who presented with PWS-like phenotype, but in whom PWS had been excluded.

Methods

The median age of the 78 patients enrolled in the study was 18.5 months, and the range was 1.4 to 324 months. Sex ratio was 1:1. All patients demonstrated PWS-like phenotype including hypotonia during infancy. We initially performed the *SNURF-SNRPN* DNA methylation test, and normal methylation results excluded the diagnosis of PWS.[8]

This study was approved by the Institutional Review Board Committees at Hokkaido University Graduate School of Medicine and National Center for Child Health and Development. The parents of the patients gave written informed consent.

DNA methylation status at the promoter region of imprinted *MEG3*, located in 14q32.2, was examined (**Figure 1**). Genomic DNA was extracted from leukocytes and treated with sodium bisulfite, and methylated allele– and unmethylated allele–specific primers were used to polymerase chain reaction amplify each allele, as described previously.[9] If aberrant DNA methylation was identified,

PWS	Prader-Willi syndrome
Upd(14)mat	Maternal uniparental disomy 14
Upd(14)pat	Paternal uniparental disomy 14

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we carried out microsatellite polymorphism analysis for 16 loci on chromosome 14 (ABI PRISM Linkage Mapping Set v2.5; Applied Biosystems, Foster City, California) with DNA from the patients and their parents (**Figure 1**). Polymerase chain reaction products were analyzed on an ABI310 automatic capillary genetic analyzer and with GeneMapper software (Applied Biosystems). If aberrant DNA methylation was identified but the patient demonstrated biparental origin of the chromosome 14s, we further examined the chromosomes for DNA methylation state, parental origin, and microdeletion in 14q32.2, as described previously.[2, 3]

Results

We identified abnormal hypomethylation at the *MEG3* promoter in 5 of 78 patients (**Figure 2**). Almost complete lack of methylation was found in 4 patients (case 1 to 4), but 1 patient (case 5) demonstrated faint methylation. Polymorphism studies demonstrated that 3 (cases 2 to 4) of the 4 patients with complete lack of *MEG3* promoter methylation had complete upd(14)mat, but 1 patient (case 1) had inherited both parental alleles (**Table I**; available at www.jpeds.com). We further examined the DNA methylation state and microdeletion or segmental upd at 14q32.3, and concluded that this patient (case 1) had an epimutation. The detailed data have been reported previously.[3] The patient (case 5) with faint *MEG3* methylation was demonstrated to have 2 maternal alleles, as well as 1 paternal allele with lower signal intensity. This indicated mosaicism of upd(14)mat (80%) and a normal karyotype (20%) (**Figure 3**; available at www.jpeds.com).

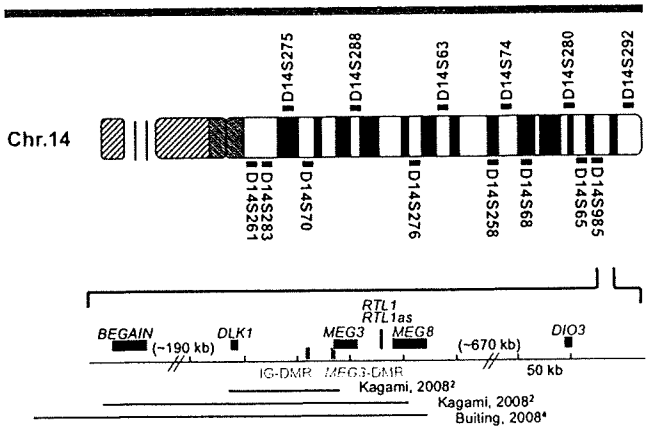

Figure 1. Schematic map of the 14q32.2 imprinted region. Loci on chromosome 14 represent markers used for microsatellite polymorphism analysis. Paternally expressed genes are shown in *blue*, maternally expressed genes in *red*, and nonimprinted genes are shown in *black*. Differentially methylated regions (DMRs) are shown in *green*. *IG-DMR*, Intergenic DMR. Reported microdeletions are demonstrated as *horizontal bars*.

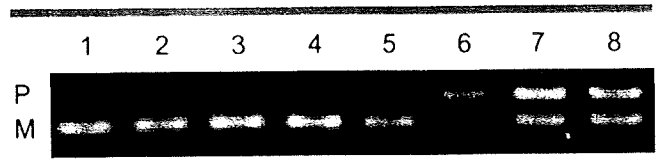

Figure 2. *MEG3* methylation test. *P*, Paternal methylated signal; *M*, maternal unmethylated signal; *1-5*, cases 1-5, respectively; *6*, paternal uniparental disomy 14; *7*, patient with PWS; *8*, normal control. Cases 1-4 show only the maternal unmethylated signal, and case 5 shows a faint paternal methylated signal.

The profiles of the patients with upd(14)mat or an epimutation are shown in **Table II**. We compared clinical features in these patients (**Table III**). All patients were referred to us during infancy because of hypotonia and motor developmental delay. Small hands and feet were also present in all patients. Prenatal growth retardation was present in all but 1 patient (case 1) who was later shown to have an epimutation. However, this patient had development of postnatal growth retardation, which was present in all patients. Premature onset of puberty was not evaluated in this study because the patients were too young. Apparent intellectual delay was only present in the patient who had upd(14)mat mosaicism (case 5). The clinical features of the patients with epimutation or with mosaic upd(14)mat were not distinct from those of the patients with full upd(14)mat.

Discussion

We detected 5 patients with upd(14)mat or epimutation at the 14q32.2-imprinted region in 78 subjects who had initially been suspected to have PWS. Mitter et al[7] reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS. However, Cox et al[10] reported that they did not find any upd(14)mat in 35 patients suspected to have PWS. Our study suggests that a significant number of patients with upd(14)mat are suspected to have PWS during infancy. To clarify how upd(14)mat and PWS share clinical features, we examined the clinical manifestations of our patients with upd(14)mat or an epimutation. All patients showed neonatal hypotonia and were referred to us during infancy. Feeding difficulty in the neonatal period and small hands and feet were also common to these patients and resembled features of PWS. It is noteworthy that all patients were referred during infancy, suggesting that upd(14)mat and PWS resemble each other, particularly during this period. Therefore upd(14)mat and related disorders, as well as PWS, should be important differential diagnoses for infants with hypotonia and feeding difficulty. Distinct features for upd(14)mat included less-specific facial characteristics, constant prenatal growth failure, and better intellectual development. Precocious puberty is not present in PWS; however, this was not evaluated in this study because the patients were not

Table II. Profiles of the patients with upd(14)mat and epimutation of 14q32.2

	Case 1	Case 2	Case 3	Case 4	Case 5
Molecular class	Epimutation	Upd(14)mat	Upd(14)mat	Upd(14)mat	Upd(14)mat (mosaic)
Age	2 y 2 m	4 y 2 m	2 y 7 m	1 y 9 m	3 y 4 m
Sex	Female	Male	Female	Female	Female
Karyotype	46,XX	46,XY	46,XX	46,XX	46,XX
Gestational age	41 w 5d	36 w 1 d	37 w 3 d	40 w 4 d	36 w
Birth weight g (SD)	3034 (0)	1955 (−2.6)	1680 (−3.3)	1858 (−2.8)	1434 (−3.9)
Birth length cm (SD)	50 (+0.7)	45.7 (−1.5)	40 (−4.0)	45 (−1.6)	39 (−3.9)
Birth OFC cm (SD)	Unknown	32 (−1.0)	30.4 (−2.0)	32 (−0.8)	30 (−2.2)
Present height cm (SD)	76.1 (−3.1)	89.5 (−2.8)	79 (−2.7)	72.5 (−3.4)	77.8 (−4.5)
Present weight kg (SD)	8.18 (−2.4)	11.6 (−2.1)	8.4 (−2.8)	6.4 (−3.7)	8.84 (−3.3)
Present OFC cm (SD)	45.2 (−1.5)	51.0 (+0.5)	48 (0)	44 (−1.8)	46.0 (−1.6)

old enough to demonstrate this feature. It is possible that when the patients get older, the clinical features of upd(14)mat may become more distinct from those of PWS.

We detected an epimutation in the 14q32.2-imprinted region, as well as upd(14)mat. The clinical features of the patient with the epimutation were grossly similar to those of patients with upd(14)mat. Thus far 5 patients with an epimutation in the paternal allele, including our patient, have been identified.[4,11] These patients exhibit clinical features indistinguishable from those with full upd(14)mat. Our patient with an epimutation demonstrated normal birth weight, but previously reported patients with an epimutation have shown intrauterine growth retardation. [4,11] Therefore normal birth weight is not a specific feature related to epimutation.

One of the patients with upd(14)mat was mosaic for upd(14)mat and normal karyotype. It is not easy to understand the pathogenesis of such a mosaic, but similar mosaicism of chromosome 15 has been reported.[12] Mosaicism for upd(15)mat and normal cell lines has been found in a patient with the PWS phenotype.[12] Similarly, our patient with mosaic upd(14)mat demonstrated typical clinical features of upd(14)mat. This could be explained by the small proportion of normal cell lines (less than 20%), or it could be that the level of mosaicism is different in each tissue. It is possible that the proportion of normal cells may be lower in the brain, which is most responsible for the phenotype of upd(14)mat.

As is clear in our series of patients, upd(14)mat phenotype can be caused by an epimutation of 14q32.2. Recently, Kagami et al[2] reported a microdeletion in 14q32.2 associated with a similar phenotype (**Figure 1**). Buiting et al[4] also reported a patient with a 1Mb deletion at 14q32.2 (**Figure 1**). Therefore upd(14)mat phenotype is associated with not only upd(14)mat but an epimutation or small deletion. This genetic complexity is similar to that of PWS. PWS is caused by paternal deletion of 15q11-q13, maternal uniparental disomy of chromosome 15, and epimutation (imprinting defect). A new name such as upd(14)mat syndrome would be appropriate to represent the entire upd(14)mat clinical features represented by upd(14)mat, epimutation of 14q32.2 and microdeletion in 14q32.2. Alternatively, Buiting et al[4] suggested the term, "Temple syndrome," because upd(14)mat was first described by Dr. I. K. Temple in 1991, who subsequently described an epimutation in 2007.[4,5,11]

Finally, it should be emphasized that the *MEG3* methylation test could detect not only upd(14)mat but an epimutation and small deletions involving *MEG3*.This is because the *MEG3* DMR that is used for the diagnostic DNA methylation test is involved in the shortest region of overlap of the microdeletions (**Figure 1**). It is therefore a powerful method for screening patients with upd(14)mat syndrome.

Table III. Clinical features in patients with upd(14)mat, epimutation and microdeletions of 14q32.2

	Present study					Previous studies		
	Case 1	Case 2	Case 3	Case 4	Case 5	Upd(14)mat (n = 35)	Epimutation (n = 4)	Microdeletion (n = 4)
Premature delivery	−	−	−	−	−	10/25	0/4	0/3
Prenatal growth failure	−	+	+	+	+	24/27	4/4	3/3
Postnatal growth failure	+	+	+	+	+	26/32	3/4	3/3
Somatic features	+	+	+	+	+	23/35	4/4	3/3
Frontal bossing	+	+	+	+	−	9/9		
High arched palate	−	+	+		+	7/9		
Micrognathia	+	+	−	+	+	5/5		
Small hands	+	+	+	+	+	24/27	4/4	3/3
Scoliosis	−	−	−	−	−	5/19		
Others								
Hypotonia	+	+	+	+	+	25/28	4/4	1/1
Obesity	−	−	−	−	−	14/34	3/4	1/4
Early onset of puberty	NA	NA	NA	NA	NA	14/16	3/4	2/3
Mental retardation	−	−	−	−	+	10/27	2/4	1/4

NA, Not applicable.
Previous studies are based on references 2, 3 and 4.

Upd(14)mat syndrome demonstrates PWS-like phenotype during infancy, and it should be considered when seeing a patient with hypotonia. The *MEG3* methylation test should be performed to identify this syndrome. ■

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ORIGINAL ARTICLE

Mucolipidosis II and III alpha/beta: mutation analysis of 40 Japanese patients showed genotype–phenotype correlation

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Mucolipidosis (ML) II alpha/beta and III alpha/beta are autosomal recessive diseases caused by a deficiency of α and/or β subunits of the enzyme *N*-acetylglucosamine-1-phosphotransferase, which is encoded by the *GNPTAB* gene. We analyzed the *GNPTAB* gene in 25 ML II and 15 ML III Japanese patients. In most ML II patients, the clinical conditions 'stand alone', 'walk without support' and 'speak single words' were impaired; however, the frequency of 'heart murmur', 'inguinal hernia' and 'hepatomegaly and/or splenomegaly' did not differ between ML II and III patients. We detected mutations in *GNPTAB* in 73 of 80 alleles. Fourteen new mutations were c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC+c.3392C>T, c.3428_3429insA, c.3741_3744delAGAA, p.R334L, p.F374L, p.H956Y, p.N1153S and duplication of exon 2. Previously reported mutations were p.Q104X, p.W894X, p.R1189X and c.2715+1G>A causing skipping of exon 13. Homozygotes or compound heterozygotes of nonsense and frameshift mutations contributed to the severe phenotype. p.F374L, p.N1153S and splicing mutations contributed to the attenuated phenotype, although coupled with nonsense mutation. These results show the effective molecular diagnosis of ML II and III and also provide phenotypic prediction. This is the first and comprehensive report of molecular analysis for ML patients of Japanese origin.
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Keywords: genotype–phenotype correlation; *GNPTAB*; *GNPTG*; I-cell disease; Japanese; mucolipidosis II alpha/beta; mucolipidosis III alpha/beta; mutation analysis

INTRODUCTION

Mucolipidosis (ML) is clinically characterized by developmental delay and dysostosis multiplex, which is partially overlapped with mucopolysaccharidoses. ML II and III are autosomal recessive diseases caused by reduced enzyme activity of *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) (EC 2.7.8.17). Targeting of newly synthesized lysosomal enzymes to lysosomes is mediated mainly by mannose-6-phosphate receptor, which recognizes the phosphate at the end of the sugar chain on lysosomal enzymes. The recognition marker is synthesized in a two-step reaction and GlcNAc-phosphotransferase acts in the first step. In patients, targeting of many lysosomal enzymes to the lysosome is impaired and levels of overflowed lysosomal enzymes are elevated in the serum and body fluids.[1]

Structural analysis of bovine GlcNAc-phosphotransferase shows that this enzyme is an $\alpha_2\beta_2\gamma_2$ hexameric peptide complex.[2] In 2000, Raas-Rothschild *et al.*[3] reported that the γ subunit is encoded by the *GNPTG* gene and contributes to the pathology of ML III gamma, formerly described as ML IIIC.[4] Recent cloning of cDNAs for α/β subunits showed that it is encoded by a single gene *GNPTAB*.[5] *GNPTAB* is located at chromosome 12q23.3, contains 21 exons and codes 1256 amino acids. The α–β boundary is located within exon 14, but the detailed mechanism of processing the precursor into α and β subunits is not clear.[6] A series of genetic-complementation studies have shown heterogeneity in ML III and the genetic relationship between ML II and III.[7–9] Mutations in *GNPTAB* cause both the severe type of ML (ML II alpha/beta, ML II, I-cell disease (MIM 252500)) and the attenuated type of ML (ML III alpha/beta, ML IIIA, Pseudo-Hurler polydystrophy (MIM 252600)).[10–12] Mutations in *GNPTG* cause the attenuated type of ML (ML III gamma, ML IIIC, ML III variant (MIM 252605)).

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We analyzed the *GNPTAB* gene in 40 Japanese ML II and III patients who had been diagnosed clinically or biochemically. Because the previously recorded clinical information was ambiguous, we reviewed the clinical records of these patients. In this article, we show the results of mutation analysis of the *GNPTAB* gene in 40 Japanese ML II alpha/beta and III alpha/beta patients and the comparison of the obtained genotype and phenotypes.

MATERIALS AND METHODS

Patients

The cases were Japanese ML II and III patients diagnosed after the 1970s to date. The diagnosis of ML was based on clinical manifestations and lysosomal enzyme activities in serum, lymphocyte and skin fibroblasts. The number of samples was 40, including 25 samples of ML II and 15 samples of ML III. In this study, all patients were probands and no siblings were included. New patients were informed about the gene test by counselors in each institute and consented. All methods in this study were approved by the ethics committee of Osaka University Graduate School of Medicine.

Clinical information

The clinical information of each patient followed in our outpatients' clinic was reviewed from the medical records at our institute. To obtain clinical information about patients followed in other institutes, we sent questionnaires to outpatients' doctors or patients' families. Many medical records had been discarded because of the long period since their death or the end of follow-up. In some cases (cases 4, 12, 13, 14 and 34), only information, such as 'ML II patient' or 'I-cell disease', was available.

Cell culture

Skin fibroblasts of patients were stored in liquid nitrogen at −196 °C. Half of each thawed sample was subjected to re-culture with Dulbecco's modified Eagle's medium (GIBCO; Grand Island, NY, USA) with 10% fetal bovine serum and anti-biotic-anti-mycotic (GIBCO). The other half of each frozen sample was directly subjected to RNA and DNA extraction.

Total RNA and genomic DNA extraction

We extracted total RNA and genomic DNA using a standard extraction kit (Isogen; Nippongene, Tokyo, Japan) from patients' peripheral blood leukocytes and/or cultured skin fibroblasts.

Reverse transcription

The obtained total RNA was subjected to reverse transcription to construct cDNA. Synthesis of cDNA was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Amplification of cDNA and genomic DNA

A cDNA fragment covering the whole coding region of *GNPTAB* was amplified by PCR in two fragments (first PCR) and each fragment was secondarily amplified in two fragments (second PCR). In other words, whole cDNA was amplified in four overlapping fragments. Six pairs of primer sequences for amplification of cDNA are listed in Supplementary 1. For genomic DNA amplification of each exon of *GNPTAB* by PCR, we used genomic primers described earlier by Kudo *et al.*[10] (primer ID; 1088, 1076, 1077, 1078, 1082, 1118, 1119, 1085, 1086, 1089, 1107, 1092, 1109, 1120, 1121, 1122, 1123, 1163, 1164, 1129, 1134, 1135, 1136, 1215, 1216, 1139, 1140, 1141, 1142, 1219 and 1259) and other newly designed primers (Supplementary 1). For the amplification of each exon of *GNPTG*, we used primers described earlier by Raas-Rothschild *et al.*[3] and other newly designed primers (Supplementary 1). PCR reactions were conducted in 20 µl reaction volumes with rTaq DNA polymerase (Takara Bio Inc., Otsu, Japan). The basic thermal profile consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 2 min and 72 °C for 2 min, with a final extension at 72 °C for 7 min. Annealing temperature was modified within 55 and 60 °C. Three microliters of each PCR mixture were run on agarose gel to ensure that only the specific product was amplified.

DNA sequencing

PCR products were purified using a standard kit (SUPREC-02; Takara Bio Inc.) to remove unnecessary primers and finally dissolved in pure water at a concentration of approximately 2–10 ng µl^{-1} water as a sequencing reaction template. DNA sequencing was carried out with the same primers as mentioned above (Supplementary 1) using the BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 3100 genetic analyzer according to the manufacturer's instructions. First, DNA sequencing was performed for cDNAs of *GNPTAB* followed by the confirmation by genomic DNA sequencing. We also analyzed the genomic DNA sequence of *GNPTG* in seven patients in whom only one mutation in *GNPTAB* was detected.

Screening for p.F374L and p.R1189X mutation by restriction fragment length polymorphism

DNA fragments containing the mutation site were amplified by PCR with primer 343/1109 (Supplementary 1) (Kudo *et al.*[10]) and 230/232 (Supplementary 1) and digested by restriction enzyme *Mbo* II or *Taq* I, respectively. PCR fragments containing p.F374L were recognized by *Mbo* II and digested from 367 to 33+16+97+221 bp. The same fragments derived from the normal allele were digested from 367 to 33+16+318 bp. With regard to PCR fragments containing p.R1189X, *Taq* I recognizes only the normal sequence and digests the fragment of 293 bp length into 205+88 bp fragments. If mutations were found by this method, DNA sequences were confirmed by genomic DNA sequencing by the same method as described above.

RESULTS

Mutations in ML II alpha/beta and III alpha/beta patients

We detected 73 mutant alleles of 80 alleles in 40 Japanese patients (Table 1). Bold letters indicate new mutations detected in this study. These include 14 new mutations and four previously detected mutations. All four known mutations were reported by a Korean group[11] and one, p.R1189X, was also reported by an Israeli group in a patient of Irish/Scottish origin.[13] The most frequent mutation was the nonsense mutation p.R1189X (c.3565C>T) and its allele frequency was 33/80 (=41.25%) in the analyzed alleles of all ML II and III patients. In 90 healthy individuals, this mutation was not found by the restriction fragment length polymorphism method described above. p.F374L was found in 1 of 25 ML II patients and 7 of 15 ML III patients, and total allele frequency was 8/80 (=10.00%). Duplication of exon 2 was found in 1 of 25 ML II patients and 5 of 15 ML III patients, and total allele frequency was 6/80 (=7.50%). No mutation was found in *GNPTG* in seven patients in whom only one mutation was detected in *GNPTAB*.

Analysis of the duplication of exon 2

First, we found mutant alleles with the structure of duplicating exon 2 in cDNA analysis (Figure 1c). We constructed forward and reverse primers within exon 2 (primer 336/335) and performed long PCR with an XL PCR Kit (Applied Biosystems). The basic thermal profile consisted of initial denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 15 s, 62 °C for 12 min, with a final extension at 72 °C for 15 min. None was amplified with normal alleles by this method because each primer runs the other way. With a mutant allele, a specific fragment was amplified and the size of the fragment was estimated as approximately 15 kbp by agarose gel electrophoresis (Figure 1d). Sequencing analysis of this fragment was performed by primer walking and the junction between introns 1 and 2 was confirmed (Figure 1b); however, we could not detect any rearrangement between exons 1 and 2 by primer walking (data not shown).

Table 1 Summary of all mutations in *GNPTAB* identified in this study

Case	Position	DNA	Protein
ML II alpha/beta			
1	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
2	**Exon 13**	**c.2089_2090insC**	**p.L697fs**
	Exon 19	c.3565C>T	p.R1189X
3	**Exon 8**	**c.914_915insA**	**p.D305fs**
	Exon 19	c.3565C>T	p.R1189X
4	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
5	Exon 13	c.2681G>A	p.W894X
	Exon 19	c.3565C>T	p.R1189X
6	**Exon 10**	**c.1120T>C**	**p.F374L**
	ND	ND	ND
7	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
8	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
9	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
10	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
11	**Exon 13**	**c.2427delC**	**p.L810fs**
	Exon 19	c.3565C>T	p.R1189X
12	**Exon 18**	**c.3388_3389insC+c.3392C>T**	**p.V1130fs**
	ND	ND	ND
13	**Exon 9**	**c.1001G>T**	**p.R334L**
	Exon 19	c.3565C>T	p.R1189X
14	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2544delA**	**p.K848fs**
15	Exon 3	c.310C>T	p.Q104X
	Exon 19	c.3565C>T	p.R1189X
16	Exon 3	c.310C>T	p.Q104X
	Exon 3	c.310C>T	p.Q104X
17	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
18	**Exon 17**	**c.3310delG**	**p.A1104fs**
	Exon 18	**c.3428_3429insA**	**p.N1143fs**
19	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
20	**Exon 21**	**c.3741_3744delAGAA**	**p.E1248fs**
	ND	ND	ND
21	**Exon 13**	**c.2544delA**	**p.K848fs**
	Exon 13	**c.2544delA**	**p.K848fs**
22	Exon 3	c.310C>T	p.Q104X
	Exon 19	c.3565C>T	p.R1189X
23	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
24	Exon 19	c.3565C>T	p.R1189X
	ND	ND	ND
25	Exon 19	c.3565C>T	p.R1189X
	Exon 19	c.3565C>T	p.R1189X
ML III alpha/beta			
26	Intron 13	c.2715+1G>A	(skip Exon 13)
	Exon 19	c.3565C>T	p.R1189X
27	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
28	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2089_2090insC**	**p.L697fs**
29	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2693delA**	**p.K898fs**
30	**Exon 2**	**Duplication exon 2**	**Frameshift**
	ND	ND	ND
31	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 13	**c.2544delA**	**p.K848fs**
32	**Exon 2**	**Duplication exon 2**	**Frameshift**
	Exon 10	**c.1120T>C**	**p.F374L**
33	**Exon 19**	**c.3458A>G**	**p.N1153S**
	Exon 19	c.3565C>T	p.R1189X
34	Intron 13	c.2715+1G>A	(skip Exon 13)
	Exon 14	**c.2866C>T**	**p.H956Y**
35	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 13	c.2681G>A	p.W894X
36	Intron 1	c.2715+1G>A	(skip Exon 13)
	Exon 14	**c.2866C>T**	**p.H956Y**
37	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
38	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
39	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X
40	**Exon 10**	**c.1120T>C**	**p.F374L**
	Exon 19	c.3565C>T	p.R1189X

Abbreviations: ML, mucolipidosis; ND, not detectable.
Bold letters indicate novel mutations detected in this study. Numbering of nucleotides starts with +1 at the first nucleotide of the initiation codon and numbering of amino acid starts with the first methionine encoded by the ATG. Descriptions about cDNA mutation and mutant protein mainly conform to the 'Nomenclature for the description of sequence variations' by Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

Polymorphisms in *GNPTAB*

The 14 polymorphisms in *GNPTAB* found in this study are listed in Table 2. Ten polymorphisms were found within the intron and four within the coding region that does not change the amino-acid residue. Not all patients were tested because these polymorphisms were found incidentally in the process of sequencing analysis of mutations. Twelve polymorphisms have been registered in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Two polymorphisms have not been reported previously.

Clinical phenotypes and genotypes

A summary of the clinical images and types of mutations of patients is shown in Table 3. We chose simple clinical manifestations, such as standing or walking, speaking, heart murmur, inguinal hernia and hepatosplenomegaly, which could be easily retrieved from old medical records or the memory of the doctors who followed them. Only the ML type was recorded in some cases but we could not obtain further information. The frequency of each clinical manifestation in the two groups is shown in Table 4.

In 33 patients, mutation was detected in both alleles. We arranged all cases into eight groups based on the type of mutation, respectively (Table 3).

Nonsense mutation contains p.Q104X, p.W894X and p.R1189X. Frameshift contains a type of mutation caused by the insertion or deletion of 1 or 4 bases, including c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC +c.3392C>T, c.3428_3429insA and c.3741_3744delAGAA. Each missense mutation, p.R334L, p.F374L, p.H956Y and p.N1153S, is

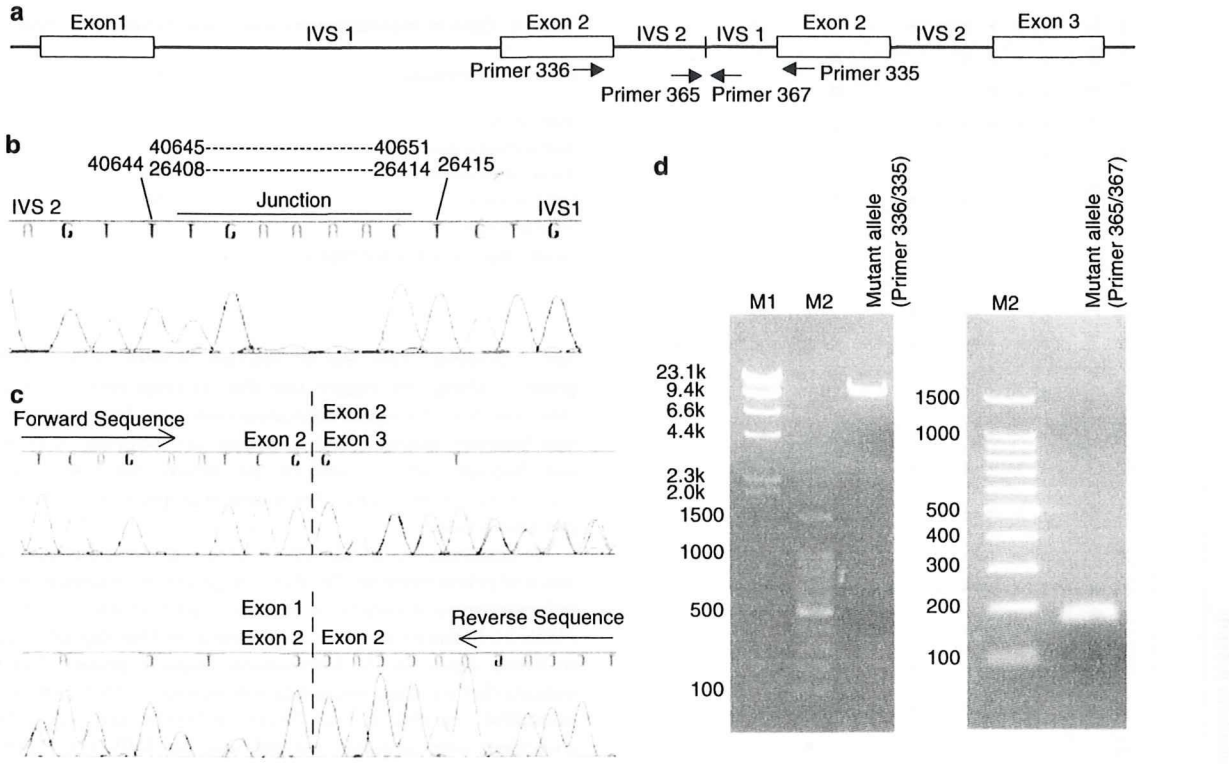

Figure 1 Summary of the duplication exon 2. **(a)** Overview of rearranged mutant allele. **(b)** Genomic sequence around the junction. XL PCR product of primer set 336/335 was used as a sequence template. Sequencing analysis of mutant allele showed that IVS 2 jumped and connected to IVS 1 through 7 overlapping bases. The number above indicates the position in reference sequence NT_019546.15. **(c)** Sequence analysis of cDNA. Forward sequencing showed overlapped signal of exons 2 and 3 next to the end of exon 2. Reverse sequencing also showed overlapped signal of exons 1 and 2 next to the end of exon 2. **(d)** Agarose gel electrophoresis of PCR products amplified by primer set 336/335 and 365/367. These primers were designed to run the other way, respectively, and only the mutant allele was amplified. Left figure indicates that the amplified fragment by primer set 336/335 was estimated to be about 15 kbp. We then sequenced the fragment by primer walking. The junction was found and primers were designed near the junction. Right figure indicates the PCR fragment amplified by primer set 365/367. Estimated fragment size was 177 bp. M1 is a ladder of λ/*Hind* III marker and M2 is a ladder of 100 bp marker (Takara Bio Inc.).

Table 2 Polymorphisms in *GNPTAB*

Position[a]	*Location in GNPTAB*	*Genomic DNA polymorphism*[b]	*cDNA*[c]
25706627	Exon 1	G>A	c.18G>A (synonymous)
25666041	Intron 2	G>T	
25665434_36	Intron 3	ATA>A (del2bases)[d]	
25665015	Intron 3	A>G	
25664898	Intron 3	T>A	
25664420_24	Intron 4	TGTGT>TGT (del2bases)	
25664372	Intron 4	C>T	
25662419	Intron 4	G>A	
25641086	Exon 13	C>G[d]	c.1800C>G (synonymous)
25640954	Exon 13	A>G	c.1932A>G (synonymous)
25640405	Exon 13	C>T	c.2481C>T (synonymous)
25637091	Intron 15	T>C	
25633304	Intron 17	T>C	
25633069	Intron 18	C>G	

[a]Position in the reference sequence NT_019546.15.
[b]Nucleotide changes from the reference sequence NT_019546.15.
[c]Numbering of nucleotides starts with +1 at the first nucleotide of the initiation codon.
[d]New polymorphisms detected in this study.

enumerated individually. Duplication of exon 2 and skipping of exon 13 are also enumerated individually, although they finally cause frameshift. Homozygotes or compound heterozygotes of the mutation are indicated by a closed circle and heterozygotes by an open circle.

DISCUSSION

In this study, several frequent mutations were detected. Nonsense mutation c.3565C>T (p.R1189X) was especially frequent in our study and its allele frequency was 41.25%. Eight of 25 ML II patients have this mutation homozygously. According to previous reports, this mutation was only found in two Korean cases[11] and in one case of Irish/Scottish origin.[13] This mutation seems to be common in eastern Asia, including Japan. It is speculated that it does not occur in a hot spot but by a founder effect, although experimental confirmation is difficult because of the unavailability of samples from the patients' parents. The secondary frequent mutation is a missense mutation c.1120C>T (p.F374L) found in eight alleles compounded heterozygously with another mutation in this study. Duplication of exon 2 was found in six alleles and this mutation occurred at the genomic level of rearrangement. Sequencing analysis of the fragment from the tail of exon 2 to the head of exon 2 showed the junction between introns 1

Table 3 Summary of clinical images and mutation types in Japanese mucolipidosis (ML) II and III alpha/beta patients

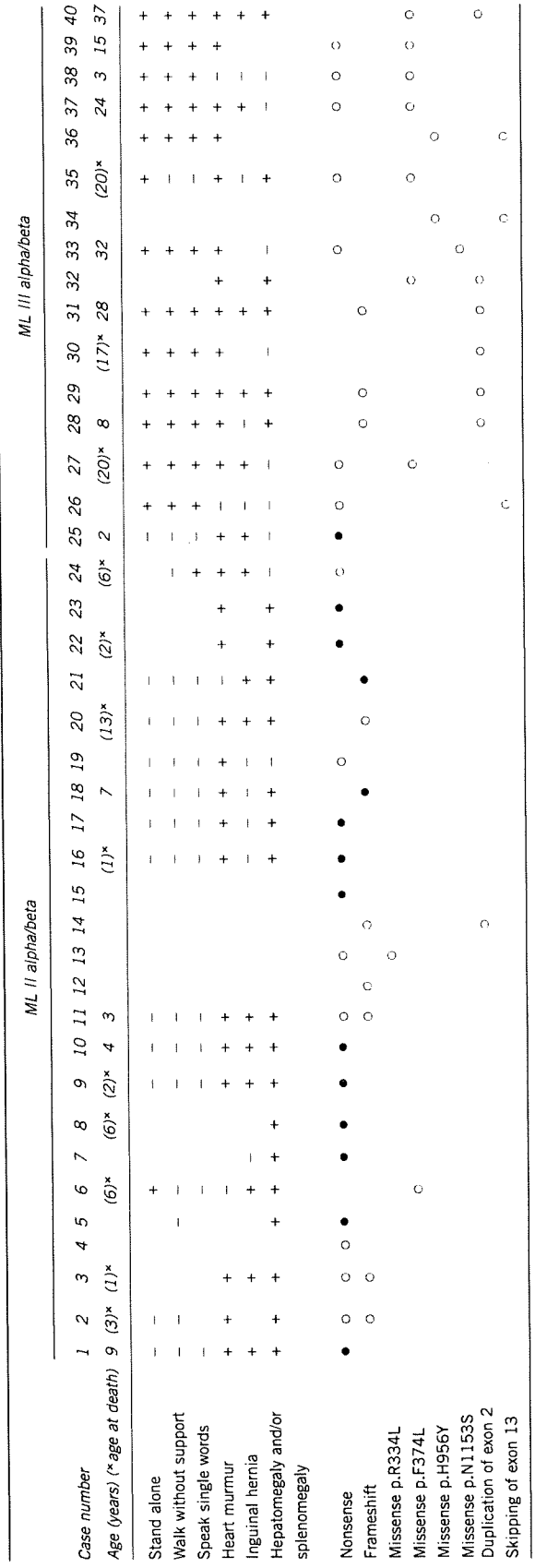

Table 4 Clinical manifestations and mucolipidosis (ML) types

Clinical manifestations	*ML II*	*ML III*
Stand alone	1/13	13/13
Walk without support	0/15	12/13
Speak single words	1/13	12/13
Heart murmur	15/17	12/14
Inguinal hernia	10/15	5/9
Hepatomegaly and/or splenomegaly	17/20	6/12

and 2; however, there was no junction between exons 1 and 2 by primer walking. We suggest that this rearrangement is not derived from insertion of a region containing exon 2 but from the recombination between introns 1 and 2 (Figure 1a). Skipping of exon 13 is also frequent and is caused by the intronic one-base substitution c.2715+1G>A mutation. This mutation is discussed in detail in the previous article.[11]

We examined the founder effects of several mutations based on the result of polymorphisms. We show the genotype frequencies of several polymorphisms in patients with/without p.F374L and c.2715+1G>A (Table 5). Reference frequencies are data from HapMap-JPT (Japanese in Tokyo, Japan) in the International HapMap project. Our results indicate that six single-nucleotide polymorphism (SNPs) (rs10778150, rs2108694, rs6539012, rs10778148, rs759935 and rs376475) are coincident with mutation p.F374L and the SNP (rs3751249) with c.2715+1G>A in Japanese ML patients. We suggest that high correspondence between the two mutations and SNPs is most likely explained by a founder effect. With regard to the other mutations, there seemed to be no relationship between the mutations and polymorphisms.

Traditionally, ML patients have been classified into severe type (as type II) and attenuated type (as type III). We examined genotype–phenotype correlation with the obtained mutational information and patient diagnosis, namely ML type. Patients with a combination of homozygotes or compound heterozygotes within nonsense mutation, p.Q104X, p.W894X and p.R1189X, showed clinically severe phenotypes. Frameshift mutations caused by insertion or deletion of 1 or 4 bases (c.914_915insA, c.2089_2090insC, c.2427delC, c.2544delA, c.2693delA, c.3310delG, c.3388_3389insC+c.3392C>T, c.3428_3429 insA, c.3741_3744delAGAA) also contribute to the severe phenotype.

On the other hand, p.F374L is considered to contribute to relatively mild clinical manifestations, except for case 6, which was reported in detail by Kojima *et al.*[14] and also by Okada *et al.*[15] (case 9 in the article). He could stand alone but could not walk and was classified into ML II. We think that this was not a typical ML II case and showed a relatively attenuated phenotype. The other cases of p.F374L, if combined with nonsense or frameshift mutation, clinically showed ML III.

Duplication of exon 2 was found in attenuated cases, except for case 14; however, we cannot discuss case 14 further because limited information was available. We consider that duplication of exon 2 contributes to the attenuated phenotype. c.2715+1G>A causes skipping of exon 13.[11] Case 26 has p.R1189X and c.2715+1G>A and shows an attenuated clinical phenotype. We consider that c.2715+1G>A also contributes to the attenuated phenotype.

Exon 2 contains 86 base sequences and exon 13 contains 1103 base sequences. The above two mutations consequently lead to frameshift. The Korean group reported skipping of exon 13 only in ML III

Table 5 Genotype frequencies of mutations and polymorphisms

Position[a] *(dbSNP id)*	*Population*	*Genotype frequency*						n
	HapMap-JPT	T/T	0	T/A	0.111	A/A	**0.889**	
25664898 (rs10778150)	Without p.F374L		0		0		**1.000**	8
	p.F374L hetero		0		**1.000**		0	2
	HapMap-JPT	C/C	0	C/T	0.119	T/T	**0.881**	
25664372 (rs2108694)	Without p.F374L		0		0		**1.000**	6
	p.F374L hetero		0		**1.000**		0	5
	HapMap-JPT	G/G	0.023	G/A	0.114	A/A	**0.864**	
25662419 (rs6539012)	Without p.F374L		0		0.143		**0.857**	14
	p.F374L hetero		0		**1.000**		0	6
	HapMap-JPT	A/A	0	A/G	0.244	G/G	**0.756**	
25640954 (rs10778148)	Without p.F374L		0		0.133		**0.867**	15
	p.F374L hetero		0		**1.000**		0	6
	HapMap-JPT	T/T	0	T/C	0.244	C/C	**0.756**	
25637091 (rs759935)	Without p.F374L		0		0		**1.000**	10
	p.F374L hetero		0		**1.000**		0	5
	HapMap-JPT	C/C	0	C/G	0.250	G/G	**0.750**	
25633069 (rs3736475)	Without p.F374L		0		0.143		**0.857**	14
	p.F374L hetero		0		**1.000**		0	4
	HapMap-JPT	C/C	**0.977**	C/T	0.023	T/T	0	
25640405 (rs3751249)	Without c.2715+1G>A		**1.000**		0		0	18
	c.2715+1G>A hetero		0		**1.000**		0	3

'*n*' means the number of patients tested for single-nucleotide polymorphisms.
Bold letters indicate major genotypes within each row.
[a]Position in the reference sequence NT_019546.15.

patients. Kudo *et al.* reported a case in which splicing mutation caused an attenuated phenotype with highly suppressed enzyme activity and suggested that splicing was especially disrupted only in these fibroblasts. Our results are consistent, although the detailed mechanisms remain unclear.

Case 33 has compound heterozygosity of p.R1189X and p.N1153S. This case shows one of the most attenuated phenotypes in this study; she could speak and walk when she was 1 year old, without hepatosplenomegaly, and she is now 32 years old and working in a workshop. p.N1153S was found in only one allele; however, it is suggested that this mutation contributes to the attenuated phenotype.

In this study, clinical severity proved to be well correlated with mutational severity. Our result supports the previous report by Bargal *et al.*[13] On the basis of information about known mutations, a patient's clinical phenotype can be estimated. Even if new mutations are found, some clinical phenotypes can be predicted by the type of mutation.

We investigated the correlation between clinical manifestations and diagnosed ML types (Table 4). 'Stand alone', 'walk without support' and 'speak single words' are considered to well correlate to the ML type. In other words, a patient who cannot stand, walk or speak has a severe phenotype and life expectancy is estimated to be limited, generally called 'ML II'. Clinical manifestations, such as 'heart murmur', 'inguinal hernia' and 'hepatomegaly and/or splenomegaly', were found in both ML II and III. Unfortunately, other various clinical manifestations, such as 'age at onset', 'bone deformity' and 'neurological findings', could not be examined because of poor information about many cases in this study. If a large-scale prospective investigation about the natural history of ML is carried out in the future, these problems may be solved.

Mucolipidosis II alpha/beta and III alpha/beta are caused by the same gene, *GNPTAB*, and the clinical phenotype shows a continuum from mild to severe. In both ML II and III, life expectancy is becoming longer owing to improved supportive therapy and care. Previously classified ML II patients can now live for more than 10 years; however, there are undoubtedly differences between typical ML II and III. It is difficult to predict the clinical phenotype from only enzymatic or biochemical characteristics.[15] From a clinical standpoint, early prediction of prognosis is necessary. We could clearly classify the former ML types from selected clinical manifestations and these ML types correlated with genotypes. This is useful for predicting prognosis to analyze mutations for treatment, including hematopoietic stem cell transplantation, especially in attenuated cases diagnosed in the early stage by molecular analysis.[16]

According to the recent report,[17] 23 different mutations have been reported in the *GNPTAB* gene causing ML II and III alpha/beta. We detected 14 new mutations in the Japanese population. Three of four other already known mutations detected this time were reported only in Korean patient. This indicates that mutations found in Asian people are restricted to the Asian region and seem not to be derived from hot spots.

This is the first and comprehensive report of molecular analysis for Japanese patients of ML. Our data showed genotype–phenotype correlations in Japanese ML II and III (alpha/beta) in particular

mutations. This result provides effective molecular diagnosis and phenotypic prediction of ML II and III (alpha/beta). Additionally, we showed that clinical severity and life expectancy are also predicted by particular clinical manifestations, including standing alone, walking without support and speaking single words.

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BCOR analysis in patients with OFCD and Lenz microphthalmia syndromes, mental retardation with ocular anomalies, and cardiac laterality defects

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Oculofaciocardiodental (OFCD) and Lenz microphthalmia syndromes form part of a spectrum of X-linked microphthalmia disorders characterized by ocular, dental, cardiac and skeletal anomalies and mental retardation. The two syndromes are allelic, caused by mutations in the *BCL-6 corepressor* gene (*BCOR*). To extend the series of phenotypes associated with pathogenic mutations in *BCOR*, we sequenced the *BCOR* gene in patients with (1)

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OFCD syndrome, (2) putative X-linked ('Lenz') microphthalmia syndrome, (3) isolated ocular defects and (4) laterality phenotypes. We present a new cohort of females with OFCD syndrome and null mutations in *BCOR*, supporting the hypothesis that *BCOR* is the sole molecular cause of this syndrome. We identify for the first time mosaic *BCOR* mutations in two females with OFCD syndrome and one apparently asymptomatic female. We present a female diagnosed with isolated ocular defects and identify minor features of OFCD syndrome, suggesting that OFCD syndrome may be mild and underdiagnosed. We have sequenced a cohort of males diagnosed with putative X-linked microphthalmia and found a mutation, p.P85L, in a single case, suggesting that *BCOR* mutations are not a major cause of X-linked microphthalmia in males. The absence of *BCOR* mutations in a panel of patients with non-specific laterality defects suggests that mutations in *BCOR* are not a major cause of isolated heart and laterality defects. Phenotypic analysis of OFCD and Lenz microphthalmia syndromes shows that in addition to the standard diagnostic criteria of congenital cataract, microphthalmia and radiculomegaly, patients should be examined for skeletal defects, particularly radioulnar synostosis, and cardiac/laterality defects.
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Introduction

The X-linked microphthalmia syndromes, 11 of which are currently defined (Table 1), comprise a clinically and molecularly diverse group of disorders, a number of which overlap with X-linked mental retardation syndromes. Lenz microphthalmia syndrome has been recognized for more than 50 years and was previously assumed to be a unitary entity. However, clinical and linkage analyses suggest that it may be aetiologically heterogeneous, linked to both Xq27-q28[1] (MCOPS1; MIM 309800) or Xp11.4[2,3] (MCOPS2; MIM 300166). Specifically, the MCOPS2 form of Lenz microphthalmia syndrome has been shown to be caused by mutation of the BCL-6 corepressor gene (*BCOR*);[3] the genetic aetiology of MCOPS1 remains unknown. The original report of Lenz described a family with variable ocular manifestations, including anophthalmia, microphthalmia and coloboma. There were numerous extraocular anomalies including mental retardation, palatal and dental anomalies, congenital heart defects, skeletal defects (affecting the fingers and clavicles), unilateral renal aplasia and cryptorchidism;[4] this phenotype substantially overlaps with other X-linked microphthalmia syndromes (Table 1). Another X-linked microphthalmia-associated condition, oculofaciocardiodental (OFCD) syndrome, is characterized by ocular defects (congenital cataracts, microphthalmia), facial anomalies (septate nasal tip, high nasal bridge, midface hypoplasia, palatal anomalies), congenital cardiac defects (atrial/ventricular septal defects, other complex heart defects), dental irregularities (canine radiculomegaly, delayed and persistent dentition, hypodontia) and skeletal anomalies (syndactyly, hammer-type flexion deformities).[5–10] All affected individuals are female, with several incidences of mother-daughter transmission.

In 2004, a sequence variant was identified within the *BCOR* gene in a single family with Lenz microphthalmia syndrome.[3] The missense mutation (c.254C>T, p.P85L) cosegregated with the disease phenotype and was not identified in more than 450 control chromosomes. It represents the sole molecular cause of Lenz microphthalmia syndrome identified to date. In the same study, *BCOR* mutations were found in all tested patients with OFCD syndrome, suggesting the two conditions were allelic. The mutation types included nonsense, frameshift, deletion and splicing mutations, a finding subsequently replicated by others. Importantly, OFCD syndrome has been shown to encompass defects of laterality, including the heart and other viscera, suggesting that *BCOR* is necessary for left–right asymmetric development and that mutations in *BCOR* may represent a source of the reported male excess of laterality defects.[11,12]

This study aims to expand our understanding of the phenotypes associated with mutations in *BCOR*, supporting the hypothesis that OFCD syndrome is solely associated with null mutations in *BCOR* and to identify additional patients with Lenz microphthalmia syndrome who carry mutations in *BCOR*. We have demonstrated that mutations in *BCOR* may be responsible for apparently isolated ocular anomalies and tested the hypothesis that *BCOR* mutations cause non-syndromic cardiac/laterality defects. The continuous accumulation of patient data allows the frequency of non-cardinal phenotypes to be estimated and improve diagnosis and treatment of patients.

Materials and methods

Direct sequencing of the *BCOR* gene

The coding exons and flanking intronic sequences of the *BCOR* gene were amplified by PCR and directly sequenced as previously reported.[3]

Table 1 Defined X-linked microphthalmia syndromes

Syndrome nomenclature and alternative names	*MIM*	*Description*[a]	*Locus*	*Gene*
MCOPS1 Lenz microphthalmia syndrome (MAA)	309800	Micro/anophthalmia, dental anomalies, skeletal/digital anomalies, mental retardation, facial dysmorphia	Xq27-q28	
MCOPS2 Oculofaciocardiodental syndrome (MAA2)	300166	Micro/anophthalmia, congenital cataracts, radiculomegaly, cardiac defects, skeletal/digital anomalies, facial dysmorphia	Xp11.4	*BCOR*
MCOPS4	301590	Micro/anophthalmia, ankyloblepharon, mental retardation	Xq27-28	
MCOPS7 Midas syndrome MLS	309810	Microphthalmia, linear skin pigmentation defects	Xp22.2	*HCCS*
MCOPCT3	302300	Microphthalmia, congenital cataract		
MCOPCB1	300345	Microphthalmia, coloboma		
FDH (Focal dermal hypoplasia) Goltz syndrome	305600	Microphthalmia, coloboma, sclerocornea, linear skin lesions, digital anomalies, mental retardation		
BRESHECK syndrome	300404	Microphthalmia, CNS anomalies, skeletal anomalies, mental retardation, renal hypoplasia		
NHS (Nance–Horan syndrome)	302350	Microcornea, cataracts, dental anomalies	Xp22.13	*NHS*
ND (Norrie disease)	310600	Microphthalmia, neuroretinal degeneration, mental retardation, sensorineural deafness	Xp11.4	*NDP*
Not assigned; proposed as part of the Renpenning syndrome spectrum	300463	Microphthalmia, coloboma, skeletal/digital anomalies, mental retardation, facial dysmorphia	Xp11.23	*PQBP1*

MCOPS1, microphthalmia, syndromic 1; MAA, microphthalmia and associated anomalies; MCOPS2, microphthalmia, syndromic 2; MAA2, microphthalmia and associated anomalies 2; MCOPS4, microphthalmia, syndromic 4; MCOPS7, microphthalmia, syndromic 7; MCOPCT3, microphthalmia, isolated, with cataract 3; MCOPCB1, microphthalmia, isolated, with coloboma 1; BRESHECK, brain anomalies, retardation, ectodermal dysplasia, skeletal malformations, Hirschsprung disease, ear/eye anomalies, cleft palate/cryptorchidism, and kidney dysplasia/hypoplasia; CNS, central nervous system.
[a]Note the large phenotypic overlap of clinical features within this group of disorders.

Semi-quantitative multiplex fluorescent PCR analysis of the *BCOR* gene

Deletions within the *BCOR* gene were detected by semi-quantitative multiplex fluorescent PCR (QMF-PCR).[13,14] All exons of the *BCOR* gene and three control genes (*DSCR1* – chromosome 21, *CFTR* – chromosome 7 and *Factor IX* – chromosome X) were amplified in two duplicate multiplex reactions (primer sequences in Supplementary Table 1). One of each primer pair was labelled with the fluorescent phosphoramidite 6-FAM dye. Amplifications were performed in 25 μl reactions using the QIAGEN Multiplex PCR kit (Qiagen, France) with 75 ng of genomic DNA and a mix of primers (concentration range 1.3–8 μM). The reaction started with an initial denaturation of 15 min at 95°C followed by 23 cycles at 95°C for 30 s/60°C for 30 s/72°C for 45 s with an increment of 3 s per cycle. Final extension was at 72°C for 10 min. PCR products were purified and processed as previously described.[14] Two control DNAs (male and female) were included in each experiment. Results were analysed by superimposing fluorescent profiles of tested patients and controls.

Fluorescent *in situ* hybridization analysis of the *BCOR* gene

To determine the proportion of cells with a deletion in the *BCOR* gene, fluorescent *in situ* hybridization (FISH) was performed as previously described.[15] Metaphase spreads of peripheral leucocytes were obtained according to standard techniques. BAC clones used in FISH experiments were provided by The Wellcome Trust Sanger Institute (Cambridge, UK). Clones localized on chromosome Xp11.4 (RP11-320G24, RP11-330L22 and RP11-429N5) were directly labelled with Cy3. Chromosomes were counterstained with DAPI. The specific signal intensity and its sublocalization along the chromosome axis were analysed using a Leica fluorescence microscope equipped with the Visilog-6 program (Noesis, Les Ulis, France).

Ethics approval

The human subject research described here was reviewed and approved by ethics committees at St Mary's Hospital (Manchester, UK), the National Institutes of Health (Bethesda,

MD, USA), Baylor College of Medicine (Houston, TX, USA), and Hôpital Henri Mondor (Créteil, France).

Results

Sequence and copy number analysis of *BCOR* in patients with OFCD syndrome

From existing literature and previous work, we have identified 33 females with OFCD syndrome.[3,5–11,16–25] In this study, two of these patients are described in further clinical detail[11] and mutational analysis of *BCOR* completed in a family (mother and daughter).[25] In addition to these four previously reported patients, we have identified a further 31 females putatively diagnosed with OFCD syndrome and found mutations in *BCOR* in all families, bringing the cohort of patients described here to 35 cases. A summary of the phenotypes and mutations identified in patients with OFCD syndrome is given in Table 2. The positions of mutations in relation to the exon sequences are shown in Figure 1. The reference sequence used for mutation numbering is given in Supplementary Data 1. The 31 novel patients described here brings the total number of reported cases of OFCD syndrome to 64. We have also sequenced three females with features of OFCD syndrome who proved negative for *BCOR* mutations; however, the phenotypes were atypical for OFCD syndrome, with a notable lack of radiculomegaly in the dental phenotype in all cases.

The mutations identified here and in other studies involve deletions of significant portions of the *BCOR* coding sequence, alterations to conserved splice acceptor sites (predicted to lead to exon skipping with concomitant frameshifts) or small insertions/deletions which cause frameshifts and generate premature stop codons; it is predicted that such premature stop codons will trigger nonsense-mediated decay of the mRNA although the generation of C-terminally truncated protein species is also possible. The observation that, in all females tested to date, X-inactivation is grossly skewed in favour of the wild-type allele (data not shown) suggests that loss of wild-type BCOR protein confers significant selective disadvantage, certainly in haematological lineages. Females with OFCD syndrome are therefore functional mosaics, with cell populations and tissues expressing either wild-type BCOR (where lack of BCOR function is presumed lethal) or no BCOR/truncated BCOR (where lack of BCOR function can be supported). Amongst the pathogenic variants are two further instances of a previously reported c.2926C>T p.R976X nonsense mutation.[3]

In this study, we report for the first time two families with individuals mosaic for a mutation in *BCOR* (OFCD XVI and OFCD XVII). In OFCD XVI, the individuals mosaic for a *BCOR* mutation have the cardinal phenotype associated with OFCD syndrome. In contrast, the mosaic individual from OFCD XVII is asymptomatic. In both families, offspring present with the cardinal OFCD syndrome phenotype and are non-mosaic for heterozygous *BCOR* mutations.

OFCD XVI Individuals XVI.1 and XVI.2 are monozygotic twin sisters, both presenting with a classical OFCD syndrome phenotype. Individual XVI.3 (the daughter of individual XVI.1) also presented with an overlapping array of features. Using a quantitative fluorescent PCR method, a deletion of at least exons 4–15 was detected in patients XVI.1 and XVI.2. However, the decrease in PCR product amount derived from the *BCOR* gene was estimated at around 75% of control peak height, rather than the 50% expected for a non-mosaic deletion (Figure 2a and b). This result was recapitulated for all *BCOR* fragments amplified and strongly suggested somatic mosaicism of this deletion. By contrast, amplification was reduced to 50% of control peak height in patient XVI.3, suggesting non-mosaic distribution of the *BCOR* deletion (Figure 2c). FISH analysis was undertaken to confirm somatic mosaicism in individuals XVI.1 and XVI.2. FISH analysis on metaphase chromosomes from both patients demonstrated a deletion corresponding to BAC clone RP11-330L22 in 52/100 metaphase preparations for each patient, with only one signal from the X chromosome (Figure 2g; data from patient XVI.1 not shown). Two distinct signals were detected in the remaining 48 leucocyte preparations (Figure 2h; data from patient XVI.1 not shown). X chromosomes were identified using an X centromere marker and by banding pattern (not shown). These results supported the hypothesis that these two individuals are mosaic for the deleted region. For patient XVI.3, 100/100 metaphase preparations generated a single signal, confirming the non-mosaic nature of the deletion in this patient (Figure 2i). Two BAC clones flanking RP11-330L22, RP11-429N5 (proximal) encompassing exons 4–15 of BCOR and RP11-320G24 (distal) were tested in patient XVI.3. Two specific signals were detected in each metaphase preparation, suggesting that these regions are not deleted in this family (the proximal RP11-429N5 probe contains sufficient sequence outside of the *BCOR* gene to permit hybridization).

OFCD XVII The proband (XVII.2), a 14-month-old girl, presented with a classical OFCD syndrome phenotype. The mother was asymptomatic and had a normal panoramic dental X-ray. Quantitative fluorescent PCR detected a reduced amount of product corresponding to exons 13 and 14, suggesting a deletion of these exons in both females. Comparison of amounts of PCR product obtained suggested that the mother (XVII.1) was mosaic for this deletion, with the amount of PCR product at 75% of the control peak height

Table 2 Clinical data and identified mutations in *BCOR* for the patient cohort presented here

Family	*Case*	*Age*	*Ocular*	*Facial/cranial*	*Cardiac*	*Dental*	*Skeletal*	*Other*	*Mutation*
OFCD I	1[a] 2[a]	34 years 8 days	Congenital cataract Microphthalmia (2) Ptosis (1) Lens dislocation (2) Optic disc dysplasia (2)	Septate nasal cartilage High nasal bridge (2)	Heart murmur (1) ASD (2) VSD (2) Pulmonary valve stenosis (2)	Unerupted secondary teeth (1) Root radiculomegaly (1) Hypodontia (1) Not recorded (2)	Hammer toes (1) Radioulnar synostosis (1)	Poor feeding (2)	c.2926C>T p.R976X
OFCD II	1[b]	23 years	Congenital cataract Microphthalmia	Septate nasal cartilage	VSD PDA MI Dextrocardia	Persistant primary teeth Unerupted secondary teeth Root radiculomegaly Hypodontia	Hammer toes	Hearing impairment	c.1539_1540insG p.P514AfsX4
OFCD III	1 2 3 4 5 6[b]	69 years 65 years 43 years 40 years 21 years 4 years	Congenital cataract Microphthalmia Glaucoma (2, 4) Phthisis bulbi (1)	Septate nasal cartilage (1, 3, 4, 5) Cleft palate (6) Not affected (2)	ASD (6) VSD (6) DORV (6) Not recorded (1, 2, 3, 4, 5)	Delayed primary dentition (6) Persistent primary teeth (1, 3, 5) Unerupted secondary teeth (1, 2, 3, 4, 5) Root radiculomegaly (1, 2, 3, 5) Hypodontia (5)	Hammer toes (1, 2, 4, 5) Second-third toe syndactyly (3, 6) Radioulnar synostosis (4, 6)	Mental retardation (6) Asplenia (6)	c.4116delGinsCT p.E1372DfsX37
OFCD IV	1 2	37 years 11 years	Congenital cataract Microphthalmia Microcornea Iris synechia (1)	Septate nasal cartilage High nasal bridge Long/narrow face (1) High arched palate (2)	Aortic valve stenosis (1) Not affected (2)	Persistant primary teeth Unerupted secondary teeth Root radiculomegaly Hypodontia Fusion of teeth (1) Duplicated teeth (2)	Hammer toes (1) Second-third toe syndactyly Lordosis	Mild mental retardation (2) Hearing impairment Vomiting/ reflux	c.4488_4497del10 p.G1497PfsX68
OFCD V	1	31 years	Congenital cataract Microphthalmia	Septate nasal cartilage High nasal bridge	ASD	Persistant primary teeth Unerupted secondary teeth Root radiculomegaly	Hammer toes Radioulnar synostosis Lumbar scoliosis		c.4512_4514 delTTGinsA p.A1506X
OFCD VI	1 2 3 4	70 years 41 years 40 years 10 years	Congenital cataract Microphthalmia (2, 4) Iris synechia (2)	Septate nasal cartilage (2, 4) Submucosal cleft palate (4) Bifid uvula (4) Not recorded (1, 3)	ASD (4) Not affected (2, 3) Not recorded (1)	Persistent primary teeth (2, 4) Unerupted secondary teeth (2, 4) Root radiculomegaly (4) Hypodontia (2, 4) Fusion of teeth (4) Duplicated teeth (2, 4) Not recorded (1, 3)	Not recorded (1, 3) Hammer toes (2) Second-third toe syndactyly (2) Scoliosis (4)	Hearing impairment (1)	c.3621delA p.K1207NfsX31
OFCD VII	1	5 years	Congenital cataract Microphthalmia	Not affected	Not recorded	Persistent primary teeth	Second-third toe syndactyly		c.4303_4307delCCATG p.P1435LfsX24
OFCD VIII	1	12 years	Congenital cataract Microcornea	Septate nasal cartilage High nasal bridge Submucosal cleft palate	Pentalogy of Fallot	Delayed dentition Root radiculomegaly Hypodontia	Second-third toe syndactyly Radioulnar synostosis	Mild mental retardation Vesicoureteral reflux	c.4200delG p.P1401RfsX83
OFCD IX	1	7 years	Congenital cataract Microphthalmia Microcornea	Septate nasal cartilage Cleft palate	ASD	Persistent primary teeth Unerupted secondary teeth Root radiculomegaly Hypodontia	Hammer toes Bilateral radioulnar synostosis		c.3848-7_3865del25 Alters conserved splice acceptor site and predicts: p.delexon9 fsX18
OFCD X	1	2 years	Congenital cataract Microphthalmia Microcornea	Septate nasal cartilage High nasal bridge Submucosal cleft palate	ASD	Delayed primary dentition	Hammer toes	Mild mental retardation	c.2428C>T p.R810X
OFCD XI	1 2	42 years 13 years	Congenital cataract Microphthalmia Glaucoma (1) Retinal detachment (1)	Septate nasal cartilage Long/narrow face (1) Simple ears (2)	ASD VSD PDA Tricuspid valve insufficiency (2)	Root radiculomegaly Hypodontia	Hammer toes (2) Short fingers (1)	Cerebral atrophy (2) ADHD (2)	c.1276_1277delCT p.L426VfsX14
OFCD XII	1	17 years	Congenital cataract Glaucoma	Septate nasal cartilage High arched palate	ASD	Root radiculomegaly	Hammer toes Second-third toe syndactyly	Mild mental retardation	Large deletion encompassing at least exons 2-15
OFCD XIII	1	5 years	Congenital cataract	Not affected	ASD	Delayed primary dentition	Hammer toes Second-third toe syndactyly	Hearing impairment	c.3649_3667 dup19 p.A1224MfsX27
OFCD XIV	1	2 years	Congenital cataract Microphthalmia Coloboma	Septate nasal cartilage Simple ears	ASD VSD	Primary dentition unaffected	Second-third toe syndactyly Limited supination at wrist Partial fusion of vertebrae (C2-C3)		c.3427_3428 insA p.S1143LfsX4
OFCD XV	1	3 years	Congenital cataract Microphthalmia	Not affected	ASD	Primary dentition unaffected	Not recorded		c.3848-1G>C p.delexon9 fsX18
OFCD XVI	1 2 3	27 years 27 years 18 months	Congenital cataract Microphthalmia	Septate nasal cartilage (1, 2) High nasal bridge (1, 2) Long/narrow face (1, 2) Not affected (3)	ASD (3) VSD (1) Not affected (2)	Delayed primary dentition (3) Delayed dentition (1, 2) Persistent primary teeth (1, 2) Root radiculomegaly (1, 2)	Second-third toe syndactyly Radioulnar synostosis (2) Scoliosis (2)		Large deletion encompassing at least exons 4-15 Somatic mosaicism (1, 2) Heterozygous (3)
OFCD XVII	1 2	33 years 14 months	Congenital cataract (2) Microcornea (2) Not affected (1)	Septate nasal cartilage (2) Long/narrow face (2) Not affected (1)	Not affected (1, 2)	Delayed primary dentition (2) Not affected (1)	Second-third toe syndactyly (2) Not affected (1)		Small deletion encompassing exons 13 and 14 c.4742-141_4976+821del1410 p.D1581GfsX15 Somatic mosaicism (1) Heterozygous (2)
OFCD XVIII	1	20 years	Congenital cataract Microphthalmia Ptosis	Septate nasal cartilage High nasal bridge Long/narrow face Cleft palate	ASD	Root radiculomegaly Hypodontia	Second-third toe syndactyly Limited supination		c.4540C>T p.R1514X

Table 2 (*Continued*)

Family	Case	Age	Ocular	Facial/cranial	Cardiac	Dental	Skeletal	Other	Mutation
OFCD XIX	1	14 years	Congenital cataract Microphthalmia Ptosis	Septate nasal cartilage High nasal bridge Long/narrow face Simple ears	Not affected	Delayed dentition Root radiculomegaly Hypodontia Fusion of teeth	Not recorded	Mild mental retardation	c.570delC p.W191GfsX25
OFCD XX	1	12 years	Congenital cataract	Septate nasal cartilage High nasal bridge Long/narrow face	Not affected	Delayed dentition Persistent primary teeth Root radiculomegaly Hypodontia	Hammer toes Second-third toe syndactyly Scoliosis		c.863delC p.P288RfsX90
OFCD XXI	1	10 months	Congenital cataract Microphthalmia	Not recorded	ASD	Not recorded	Not recorded		c.2926C>T p.R976X
Lenz I	1	7 years	Microphthalmia	Narrow forehead Simple ears	ASD	Not affected	Multiple partial finger syndactyly Fifth finger clinodactyly Radioulnar synostosis	Mental retardation Hypospadias	c.254C>T p.P85L

Patients are represented by two numbers: the first indicates the family, the second indicates the individual case. Features specific to individuals within a family are indicated in parentheses ( ). Ages are at the time of examination.
ASD, atrial septal defect; VSD, ventricular septal defect; MI, mitral valve insufficiency; PDA, patent ductus arteriosus; DORV, double outlet right ventricle; ADHD, attention deficit hyperactivity disorder.
[a]Full phenotypes in McGovern *et al*.[25] *BCOR* mutation identified in this study.
[b]Brief phenotype and mutation in Hilton *et al*.[11] Full phenotype of individual and family members described in this study.

(Figure 2d). Amplification from exons 8 and 9 were at the control intensity. Amplification from proband XVII.2 was reduced to 50% for exons 13 and 14, suggesting a non-mosaic distribution of this deletion (Figure 2e). PCR amplification and sequencing of a region spanning the proposed deletion region was performed to refine the deletion to 1410 bp from intron 12–14 (Figure 2f).

Clinical features of OFCD syndrome

The cohort presented here comprises 31 new females and four previously described females with mutations in *BCOR* (35 patients). However, one female (XVII.1) is asymptomatic, despite proving mosaic for a *BCOR* mutation, and thus is excluded from the phenotypic evaluation, bringing the cohort of symptomatic females to 34. A summary of the frequency of phenotypes within the 34 symptomatic females is given in Table 3.

Ocular OFCD syndrome is associated with ocular defects and the cardinal manifestation of congenital cataract is present in each individual in the new cohort presented here (34/34). In addition to congenital cataract, patients displayed an array of eye anomalies, most frequently microphthalmia and/or microcornea in 82% of patients (28/34; Figure 3a and b).

Facial In our cohort, 31 patients were examined for facial anomalies and a facial phenotype was recorded in 26 patients. The most specific facial manifestation is septate nasal cartilage, observed in 96% (25/26) of affected patients (Figure 3a and b), often associated with a high nasal bridge and long and/or narrow face. Many of the patients had palatal anomalies, including cleft palate, high-arched palate and bifid uvula (31%; 8/26).

Cardiac Where examined, the majority of patients in the cohort presented here have a congenital heart defect (74%; 20/27), with 85% (17/20) presenting with a septal defect, most commonly an atrial septal defect. Further cardiac anomalies such as pentalogy of Fallot, double-outlet-right-ventricle, valve insufficiencies and patent ductus arteriosus are observed, suggesting a role for *BCOR* in multiple cardiac processes.

Dental In this cohort, 30 patients were examined for dental defects. Owing to age, data corresponding only to primary dentition were available for eight patients. Of these, 75% (6/8) have a dental phenotype of delayed primary dentition and/or persistent primary teeth. Two patients have thus far unaffected primary dentition but it is anticipated that secondary dentition will be affected. Data regarding both primary and secondary dentition are available for 22 patients in this cohort and all have a variety of dental anomalies associated with OFCD syndrome, that is, delayed/persistent dentition with multiple

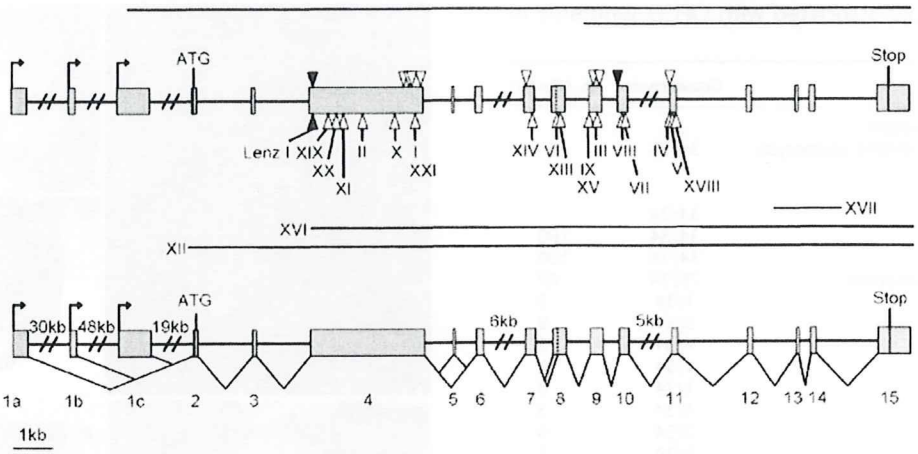

Figure 1 Location of reported mutations within the *BCOR* gene. Arrowheads above the diagram indicate mutations described in previous reports. Mutations indicated below the diagram are those identified in this study (see Table 2). Arrowheads in white indicate mutations corresponding to females with OFCD syndrome. Arrowheads in grey indicate mutations corresponding to males with Lenz microphthalmia syndrome. Arrowhead in black indicates an in-frame deletion occurring in a female with OFCD syndrome. Deletions are indicated by solid horizontal lines.

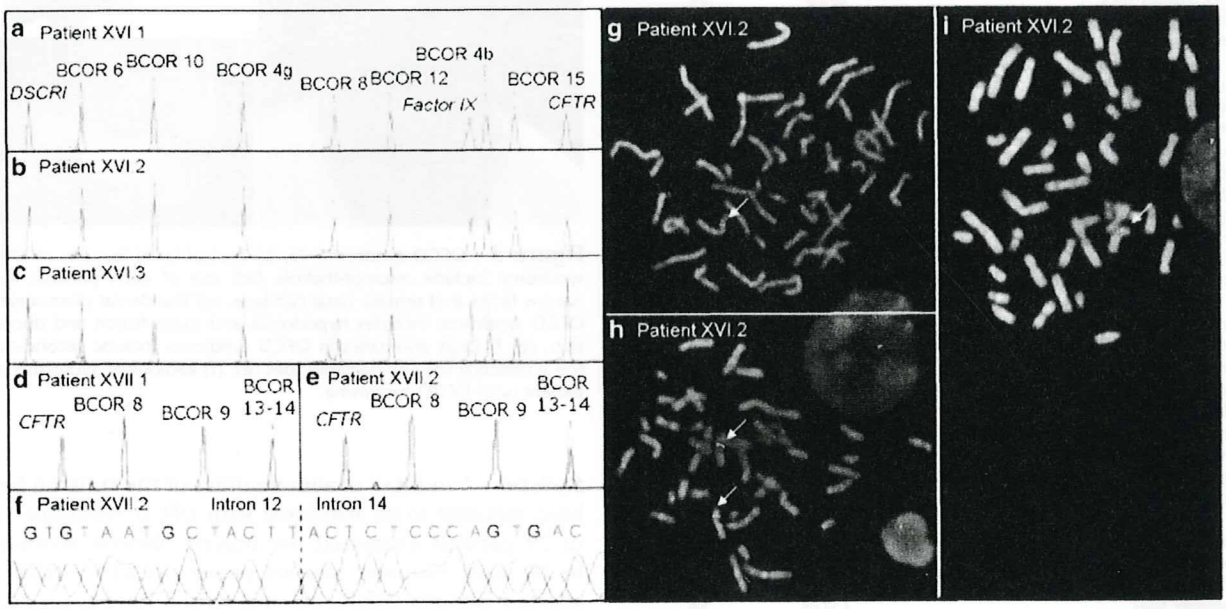

Figure 2 Evidence for somatic mosaicism in individuals XVI.1, XVI.2 and XVII.1. (**a**–**e**) QMF-PCR fluorescent spectra obtained for fragments of the *BCOR* gene and control genes, with patient peaks in red and control subject peaks in blue. Patient XVI.1 (**a**), patient XVI.2 (**b**) and patient XVII.1 (**d**) display diminished peak intensity to approximately 75% of the control peak intensity, suggesting mosaicism of the deleted region. Patient XVI.3 (**c**) and patient XVII.2 (**e**) display diminished peak intensity to approximately 50% of the control peak intensity, suggesting a non-mosaic deletion. (**f**) Sequence chromatogram of patient XVII.2, confirming a deletion between introns 12 and 14, resulting in loss of exons 13 and 14. (**g**–**i**) FISH analysis using BAC clone RP11-330L22, encompassing the *BCOR* gene, with signals indicated by white arrows. Data for patient XVI.1 are equivalent to patient XVI.2. Patient XVI.2 (**g**, **h**) displays a single signal in approximately 50% of metaphase preparations (**g**) whereas the remaining preparations show two specific signals (**h**), confirming that these individuals are mosaic for a deletion of the *BCOR* gene. For patient XVI.3 (**i**), a specific single signal was observed in 100% of metaphase preparations, confirming that the deletion of the *BCOR* gene is non-mosaic and present in all cells.

unerupted teeth, root radiculomegaly and absent/duplicated/fused teeth (100%; 22/22; Figure 3c). Root radiculomegaly, a unique and cardinal diagnostic feature of OFCD syndrome, is present in 91% (20/22) of patients, with diagnosis limited in the remaining two patients by the unavailability of a dental X-ray.