LETTER TO JMG

Clinical and molecular analysis of Mowat-Wilson syndrome associated with *ZFHX1B* mutations and deletions at 2q22–q24.1

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Hirschsprung disease (HSCR), a clinically complex syndrome often associated with a combination of mental retardation, microcephaly, and characteristic facial features, is genetically heterogeneous.[1–10] Recently, mutations in *ZFHX1B*, which encodes Smad-interacting protein 1 (SIP1), were identified by our group[11] and Cacheux *et al*[12] in patients with t(2;13)(q22;q22) and t(2;11)(q22.2;q21), respectively. These patients had clinical profiles consistent with a dominant form of the disease (Mowat-Wilson syndrome: MIM 235730).[9 13] These and subsequent articles have demonstrated a link between patients with nonsense and frameshift mutations of the gene and the presence in these patients of the following clinical findings: profound mental retardation, delayed motor development, and specific facial features, including hypertelorism, a broad nasal bridge, strabismus, a pointed chin, prognathism, a nose with a prominent columella, and posteriorly rotated ears. Often associated with these features were microcephaly, epilepsy, congenital heart disease, HSCR, and midline defects such as agenesis or hypoplasia of the corpus callosum and hypospadias.[13–19] This variety of clinical features is observed not only in patients with nonsense and frameshift mutations but also in patients harbouring deletions involving *ZFHX1B* at 2q22,[9 11 15 20] including a previously reported isolated case.[6] Thus, *ZFHX1B* clearly plays a critical role in the manifestation of clinical features of Mowat-Wilson syndrome. However, several points remain to be elucidated: (1) How broad are the clinical features in patients with *ZFHX1B* mutations and deletions? (2) What are the underlying molecular mechanisms by which the deletion of *ZFHX1B* at 2q22 affects the clinical phenotype? (3) What kind of clinical features might appear if the deletions were extended to include 2q23–q24.1? To address these questions, we have characterized the clinical features and underlying molecular basis of 27 cases with mutations or deletions in *ZFHX1B*, including 12 previously reported cases comprising 10 mutations, one deletion, and another case that had previously been found to have neither a deletion nor a mutation in the gene.

Key points

- We identified five novel nonsense and frameshift mutations in one allele of *ZFHX1B* from patients with Mowat-Wilson syndrome. One of the mutations was an insertion of 382 bp of the AluYa5 sequence at nucleotide number 1169.
- Two novel symptoms, pulmonary artery sling and septum of the vagina, were observed in patients with nonsense or frameshift mutations in *ZFHX1B*. Repeated vomiting attacks observed in five patients might be related to one aspect of epilepsy, as these attacks can be controlled with anticonvulsants.
- Using a multiplex PCR method, we identified 0.2–10.42 Mb deletions at 2q22–q24.1, including *ZFHX1B*, in eight patients. Nucleotide sequence analyses demonstrated no sequence similarity for the regions on the two sides of the breakpoints in five patients.
- All patients examined thus far have a paternal deletion and present clinical features that closely overlap those of cases with nonsense and frameshift mutations resulting from deletions of up to about 5 Mb. However, two patients with large deletions (10.42 and 8.83 Mb) had significantly delayed psychomotor development. One of them also presented with a cleft of the hard palate and complicated heart disease, features that have not been reported in patients with Mowat-Wilson syndrome.

METHODS

Clinical studies

Informed consent was obtained from all participants in accordance with institutional guidelines for human materials and subjects. Evaluation was by review of medical records and physical examination, including findings for mental retardation, developmental delay, microcephaly, facial dysmorphism, epilepsy, and Hirschsprung disease, as well as CT and/or MRI brain images. The subjects participating in this study were named S1 to S42. Subjects who are not listed in the tables, such as S12, have not been identified with an abnormality in *ZFHX1B*, or the analysis of that patient has not yet been completed.

Mutational analysis of *ZFHX1B*

To screen for mutations, DNA fragments including each exon of *ZFHX1B* from patients and normal controls were amplified by PCR[14] and subjected to direct sequencing analysis.[21] To confirm mutations detected in patients, PCR products were subcloned into pGEM-T Easy (Promega) and sequenced.

Abbreviations: GER, gastroesophageal reflux; HSCR, Hirschsprung disease; PAS, pulmonary artery sling; PDA, patent ductus arteriosus; TOF, tetralogy of Fallot

Chromosome 2 microsatellite analysis

Microsatellite analyses were performed as described previously.[11] The polymorphic loci used for haplotype analysis around *ZFHX1B* on chromosome 2q were the following: D2S129, D2S2149, D2S122, D2S132, D2S381, D2S2301, D2S151, D2S222, D2S356, D2S2299, and D2S142 (in order from centromere to telomere, according to the NCBI *Homo sapiens* Map View).

Chromosomal deletion analysis using multiplex PCR

To determine regions of deletion on chromosome 2q22–q24.1 in patients, we established a quantification method for determining the dosage of genomic DNA using multiplex PCR. *CCNT2*, which encodes Cyclin T2 and maps about 10 Mb centromeric to *ZFHX1B*, was used as a quantitative control gene with both alleles present, because deletion of *CCNT2* was not observed in any of the patients examined. Specific primers (sense: 5′-GACAGGTATCAGAGACACCA-3′ and antisense: 5′-ATTTTAGAGGTACTGGCGCAG-3′) were designed to amplify a 140 bp DNA fragment in exon 9 of *CCNT2*. Another pair of primers was also designed to amplify approximately 200 bp genomic DNA fragments from every region at 2q22–q24.1 so that repetitive sequences such as Alu and L1 were not selected. Aliquots (50 ng) of genomic DNA from each patient and a normal control subject were amplified by PCR in a total volume of 20 µl, each containing 0.3 µM in total of two pairs of primers, one for a specific region at 2q22–q24.1 and the other for *CCNT2*, 30 µM of each dNTP, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 U of AmpliTaq-Gold (Applied Biosystems), and 0.2 µl of [α-^{32}P]dCTP (80 kBq: 111 TBq/mmol). The ratios of the concentrations of the two primer pairs targeted to the specific region at 2q22–q24.1 and *CCNT2* varied from 1 to 3, depending on the sequences chosen. PCR samples were preheated at 94℃ for 10 min, and from 18 to 24 PCR cycles were performed. Each cycle consisted of 30 s denaturation at 94℃, 30 s annealing at 58℃, and 30 s extension at 72℃. The PCR products were electrophoresed on 8% polyacrylamide gels and quantification of radioactivity was performed with a BAS 1800 image analyzer (Fujifilm). Ratios of radioactivity of genomic DNA at 2q22–q24.1 versus *CCNT2* were determined for patients (Rp) with respect to normal controls (Rc). We previously reported a patient (S1) with an unbalanced reciprocal translocation t(2;13)(q22;q22) and an approximately 5 Mb deletion containing *ZFHX1B* at 2q22.[11] Using genomic DNA from this patient and five normal controls as templates, we performed multiplex PCR and determined the gene dosage of *KYNU*, which encodes kynureninase, and *ZFHX1B*, both located in the deletion region at 2q22 of this patient. When PCR was performed for between 18 and 24 cycles, the ratios (Rp/Rc) for both genes from S1 ranged from 0.3 to 0.6 (data not shown). Thus, when amplified genomic DNA was included in the region of deletion, Rp/Rc ratios typically varied from 0.3 to 0.6, whereas values for DNA without deletion ranged from 0.9 to 1.1. When ratios were between 0.6 and 0.9, new primer pairs were chosen for an adjacent region and multiplex PCR was repeated to determine whether the amplified genomic DNA was deleted. *ZFHX1B* spans approximately 132 kb of the human reference genomic contig NT_005058 in the reverse orientation, and consists of 10 exons and nine introns. The following primer pairs were designed for screening the deletion of *ZFHX1B* by multiplex PCR: exon 1 (sense: 5′-GTCCGTCCCCAGGTTTG TGT-3′ and antisense: 5′-AAAGCATGAAGAAGCCGCGA -3′); exon 3 (sense: 5′-TGTAGTGGACACAGGTTCTG-3′ and antisense: 5′-TAGAATCTCGTTGTTGTGCCA-3′); exon 6 (sense: 5′-TGCCACCTGGAACTCCAGAT-3′ and antisense: 5′-ATATC GTTTCTCTAAGGGGTTA-3′); and exon 10 (sense: 5′-ACTCGC AGCACATGAATCAC-3′ and antisense: 5′-CTCCTTCTCGCTCT CGCCA-3′).

Determination of the breakpoints of chromosomal deletions

Multiplex PCR was repeatedly performed to limit the region of deletion breakpoints in eight patients. Finally, each breakpoint was confined to a region containing a few kb of genomic DNA within a single BAC clone. Sense and antisense primers were designed for centromeric and telomeric adjacent regions of the breakpoint (fig 1A) and 50 ng of genomic DNA from each patient and a normal control subject were amplified by PCR using these primers to detect deletions.

RESULTS

Clinical features and *ZFHX1B* mutations detected in patients with Mowat-Wilson syndrome

We identified five novel nonsense and frameshift mutations (390fs430X, 465fs467X, 286fs293X, R302X, Q497X) and a nonsense mutation, R343X, reported recently[19] in one allele of *ZFHX1B*, in addition to those in the 10 patients described in detail earlier (table 1). It should be noted that a 382 bp AluYa5 sequence was inserted at nucleotide number 1169 in S15. The previously reported R695X mutation[11 14 17] was also found in three cases (S21, S32, and S38). Clinical features for

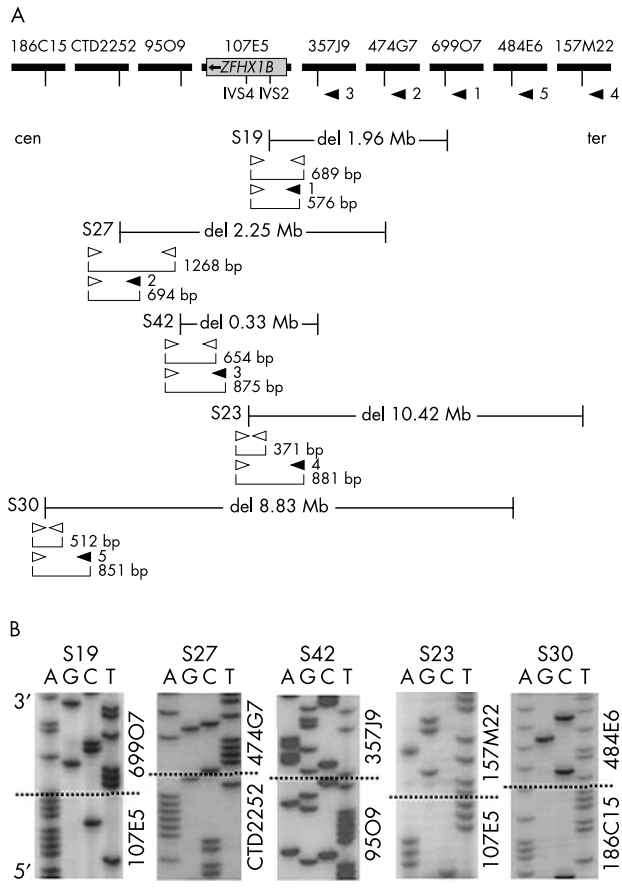

Figure 1 Determination of nucleotide sequences of breakpoints for five patients. (A) Location of the sense (▷) and antisense (◁ and ◀) primers used for PCR amplification are indicated in the BAC clones. ▷ ◁, PCR amplification of genomic DNA within BAC clones. ▷ ◀, PCR amplification of genomic DNA between two BAC clones. (B) Nucleotide sequences of breakpoints of deletions from five patients. Broken lines indicate the breakpoints. Two BAC clones including the breakpoint of the deletion are shown on the right.

all 19 patients are summarized in table 1. All subjects, except for S18, who has been described previously and harboured a single amino acid deletion (N99del),[22] demonstrated severe mental retardation, facial dysmorphism, and delayed motor development. Hirschsprung disease was present in about half the cases (9/19), microcephaly in 16/19, and epilepsy in 15/19. Congenital heart disease and a partial or complete defect of the corpus callosum were observed in nine and six patients, respectively. Defects of midline structures, such as hypospadias, cryptorchism, or hypertrophic pyloric stenosis (HPS)[15] were found in eight patients. Pulmonary artery sling (PAS), a rare anomaly in which the left pulmonary artery, arising from the right pulmonary artery, forms a vascular ring around the trachea, causing tracheal compression,[23] was observed in patient S28. This is the first case of PAS associated with a *ZFHX1B* mutation, and was complicated by patent ductus arteriosus (PDA) and congenital tracheal stenosis. Likewise, a septum of the vagina was observed in S38, the first such defect seen together with a *ZFHX1B* mutation. The affected individual also had periodically repeated vomiting attacks since 7 months of age. Initially, we believed her vomiting to be caused by a surgical problem with gastroesophageal reflux (GER) after Ramstedt pyloromyotomy. Therefore, Nissen's fundoplication and percutaneous endoscopic gastrostomy were performed at 8 months of age. Despite this treatment, her vomiting did not decrease. Thus, we suggested the repeated vomiting attacks might be related to her epilepsy, although detailed examination of her EEG was not performed during the attacks. Currently, the attacks are well controlled with sodium valproate and chlorpromazine. It should be noted that S4, S15, S19, and S31 suffered from the same repeated vomiting attacks.

Clinical features associated with deletions of 2q22–q24.1

The clinical features of eight patients with deletions including *ZFHX1B* are summarized in table 2. In six cases (S1, S3, S19, S27, S41, and S42), the features were quite similar to those of patients with *ZFHX1B* mutations (table 1); patient S3 had been previously shown to be negative for any abnormality in

Table 1 Clinical features and *ZFHX1B* mutations in patients with Mowat-Wilson syndrome

Patients (sex, age)	Nucleotide change (exon)	Amino acid change	HSCR	MR	EP	Microcephaly	Face	Walking age	Heart disease	Brain abnormality (CT/MRI findings)	Others	Refs.
S2 (F, 20 years)	1645A>T (8)	R549X	+	++	−	+	+	3 years and 6 months	PDA	ACC	Exotropia	11, 14, 17
S4 (M, 25 years)	2083C>T (8)	R695X	+	++	+*	+	+	8 years	−	NE	HPS	11, 14, 17
S5 (M, 25 years)	1173del4 (8)	392fs394X	+	++	+	+	+	5 years and 3 months	PDA	WNL		11, 14, 17
S6 (M, 28 years)	2083C>T (8)	R695X	c	++	+	+	+	8 years	−	VM (mild)	Exotropia	14
S7 (M, 30 years)	2083C>T (8)	R695X	c	++	+	+	+	4 years	VSD	WNL	Cryptorchism, esotropia	14
S8 (M, 26 years)	2083C>T (8)	R695X	c	++	+	+	+	2 years and 6 months	−	WNL		14
S9 (M, 27 years)	760insCA (6)	254fs262X	−	++	+	+	+	2 years	−	WNL		14
S10 (F, 6 years)	272delG (3)	91fs107X	−	++	−	+	+	4 years and 8 months	−	HCC (moderate), cortical atrophy, VM (moderate)		14
S11 (M, 3 years)	2178del2 (8)	727fs754X	c	++	+	+	+	3 years and 3 months	−	HCC (moderate)	Exotropia	14
S14 (M, 4 years)	1027C>T (8)	R343X	+	++	−	+	+	−	ASD, PS	NE	Hypospadias	
S15 (M, 5 years)	1169ins382 (8)	390fs430X	−	++	+†	+	+	−	−	HCC (mild)		
S18 (F, 44 years)	296del3 (3)	N99del	−	+	−	−	−	1 year and 2 months	−	WNL		22
S21 (M, 6 years)	2083C>T (8)	R695X	+	++	+	+	+	−	PDA	NE	Hypospadias	
S24 (F, 11 years)	1395del14ins19 (8)	465fs467X	+	++	+	+	+	3 years	PDA	WNL		
S28 (M, 10 years)	857delAG (7)	286fs293X	c	++	+	+	+	6 years	PAS, PDA	ACC	Cryptorchism, stenosis of trachea	
S31 (M, 7 years)	904C>T (7)	R302X	+	++	+*	+	+	5 years and 2 months	−	Cavum septi pellucidi	Cryptorchism	
S32 (M, 2 years)	2083C>T (8)	R695X	+	++	+	−	+	−	PDA, VSD	HCC (mild), cortical atrophy, VM (mild)	Cryptorchism, HPS, hypospadias	
S34 (M, 28 years)	1489C>T (8)	Q497X	−	++	+	−	+	2 years	−	NE		
S38 (F, 3 years)	2083C>T (8)	R695X	+	++	+*	+	+	−	PDA	WNL	Septum of vagina, HPS, exotropia	

*Showed repeated vomiting attacks in addition to epileptic convulsions; †showed repeated vomiting attacks only.
(+), Presence of clinical signs; (−), absence of clinical signs; (c), shows constipation.
ACC, agenesis of corpus callosum; ASD, atrial septal defect; EP, epilepsy; HCC, hypoplasia of corpus callosum; HPS, hypertrophic pyloric stenosis; HSCR, Hirschsprung disease; MR, mental retardation; NE, not examined; PAS, pulmonary artery sling; PDA, patent ductus arteriosus; PS, pulmonary stenosis; VM, ventriculomegaly; VSD, ventricular septal defect; WNL, within normal limits.

ZFHX1B.[11] Two patients (S23 and S30) who were shown to have chromosomal deletions by cytogenetic analysis, had midline facial defects (a broad nasal bridge for S23 and a cleft of the hard palate for S30), complicated congenital heart disease (tetralogy of Fallot for S23 and DORV, PA, AVSD, hypoLV, left-PDA, and RAA for S30), and severe developmental delay, which is expected to preclude them from sitting without support (fig 2A, C). Tetralogy of Fallot (TOF) has been observed in three patients having nonsense or frameshift mutations in *ZFHX1B*.[19] One of our cases (S23) has experienced several anoxic spells since the age of 6 months. These could be well controlled with calteolol (0.2 mg/kg) and phenobarbital (5 mg/kg) for some time, but the spells eventually returned. Finally, a right-modified Blalock-Taussig shunt for TOF was performed at the age of 1 year and 10 months. Another patient (S30) underwent a left-modified Blalock-Taussig shunt and PDA ligation at the age of 34 days, because of PDA-dependent pulmonary high flow. This patient also exhibited severe constipation and abdominal distension at birth. However, during treatment for congenital heart disease, the patient's abdominal symptoms spontaneously disappeared. Thus, further examination of HSCR was not performed due to the patient's severe general condition.

Determination of BAC clones containing deletion breakpoints

Using the multiplex PCR method, five patients (S3, S19, S27, S41, and S42) with features consistent with *ZFHX1B* truncation mutations were newly shown to have deletions including a part or all of *ZFHX1B*, in addition to S1, who had been previously reported.[11] By repeating many sets of multiplex PCR, we finally determined both sides of the breakpoint in a single BAC clone from each patient as follows (BAC clones containing the centromeric side of the breakpoint, and the telomeric side of the breakpoint): S1 (RP11-257N13, RP11-699O7); S3 (RP11-95O9, RP11-107E5); S19 (RP11-107E5, RP11-699O7); S27 (CTD-2252P21, RP11-474G7); S41 (RP11-4C18, RP11-514A9); and S42 (RP11-95O9, RP11-357J9) (data not shown). We determined next the breakpoints in two patients (S23 and S30) with large deletions detectable by cytogenetic analysis and severe developmental delay (table 2) compared to patients with nonsense and frameshift mutations in *ZFHX1B* using the same method. BAC clones containing the centromeric and telomeric side of the breakpoint of deletion in one allele were (RP11-107E5, RP11-157M22) for S23 and (RP11-186C15, RP11-484E6) for S30 (data not shown). As seen previously for S1,[11] microsatellite analysis demonstrated that the deletion occurred in the paternal allele in the four newly analyzed patients (fig 3).

Determination of nucleotide sequences at breakpoints

To evaluate the molecular mechanisms underlying the deletions at 2q22–q24.1, the precise sequences at the breakpoints were determined. Further sets of multiplex PCR were conducted to limit the regions of both breakpoints as close as possible for seven patients other than S1, who has an unbalanced reciprocal translocation. We performed PCR amplification of the DNA fragments including the breakpoint using primer pairs located on each side of the breakpoint. In five cases, fragments could be amplified and sequenced directly, and the precise deletion sizes were determined to be 1.96, 2.25, 0.33, 10.42, and 8.83 Mb (fig 1). Nucleotide sequence analysis around the breakpoint demonstrated that one deletion in patient S27 occurred within an L1 repeat-sequence region. However, the other patients' breakpoints occurred in regions without any specific repeating sequences. Moreover, there was no sequence similarity for the regions on the two sides of the breakpoints in any of the five patients.

DISCUSSION

In the present study, we focused on 27 patients with heterozygous changes in *ZFHX1B*, including mutations and chromosomal deletions of part or all of the gene. Those with nonsense or frameshift mutations in *ZFHX1B* consistently presented with severe mental retardation, delayed motor development, and facial dysmorphism in various combinations with microcephaly, epilepsy, HSCR, congenital heart diseases, and abnormal brain findings, including agenesis of the corpus callosum. In addition to these clinical features, non-specific findings including vaginal septum, HPS, and hypospadias were encountered, likely caused by failure of midline structures to form.[15] Repeated vomiting attacks suggestive of epilepsy were observed in five cases.

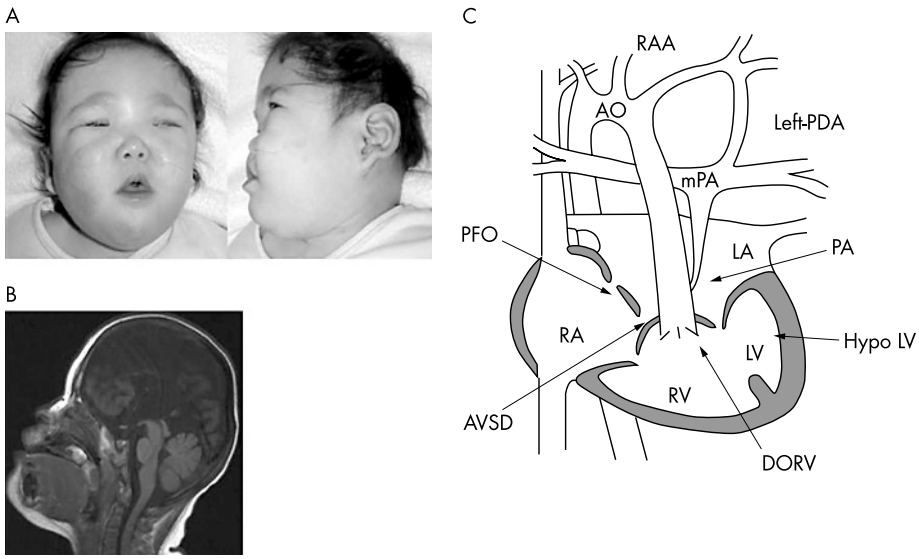

Figure 2 Clinical features of S23 and S30. (A) S23 showing hypertelorism and a broad nasal bridge. (B) MRI (T1-weighted sagittal image) of the head of S23 showing microcephaly with a reduced brain volume and agenesis of the corpus callosum. (C) A schematic illustration of the congenital heart diseases of S30. In S30, the aorta and pulmonary artery (mPA) both arise from the right ventricle (DORV). It should be noted that S30 also has cardiac malformations including PA, RAA, left-PDA, PFO, AVSD, and hypoLV.

Table 2 Clinical features and 2q22–q24.1 deletions in patients with Mowat-Wilson syndrome

	Patient							
	S1*	**S3†**	**S19**	**S27**	**S41**	**S42**	**S23**	**S30**
Age, sex	9 years, female	26 years, female	3 years, female	3 years, male	18 years, male	17 years, male	3 years, female	1 year, female
Karyotype	46,XX, t(2;13)(q22;q22)	46,XX	46,XX	46,XY	46,XY	46,XY	46,XX, del2q23.1 –24.2	46,XX, del2q22.3–24.1
Size of deletion	~5.1 Mb	~0.2 Mb	~2.0 Mb	~2.3 Mb	~2.8 Mb	~0.3 Mb	~10.4 Mb	~8.8 Mb
ZFHX1B deletion	All	Exons 3–10	Exons 1–2	All	All	All	Exons 1–4	All
Family history	Nothing of note	Nothing of note	Nothing of note	Nothing of note	Nothing of note	Nothing of note	Nothing of note	Nothing of note
Perinatal								
Gestational age	40 weeks 4 days	41 weeks 3 days	41 weeks 1 day	38 weeks 3 days	40 weeks 0 days	40 weeks 1 day	36 weeks 2 days	38 weeks 2 days
Labor	S. vaginal	S. vaginal	S. vaginal	Caesarean section	S. vaginal	S. vaginal	Caesarean section	Caesarean section
Body weight	3000 g (−0.6 SD)	3200 g (+0.1 SD)	3320 g (+0.4 SD)	3338 g (+0.6 SD)	2720 g (−1.5 SD)	3140 g (−0.4 SD)	1968 g (−1.5 SD)	2848 g (−0.1 SD)
HC	31.5 cm (−1.5 SD)	32.0 cm (−1.3 SD)	31.8 cm (−1.5 SD)	33.0 cm (−0.1 SD)	33.0 cm (−0.5 SD)	32.0 cm (−1.2 SD)	27.8 cm (−1.6 SD)	32.2 cm (−0.6 SD)
Development								
DQ (age)	7 (9 years)	7 (20 years)	27 (2 years 9 months)	24 (3 years)	7 (18 years)	5 (17 years)	NE	NE
Head control	4 months	5 months	4 months	3 months	5 months	3 months	1 year 3 months	–
Sitting	1 year 6 months	9 months	1 year 2 months	2 years 4 months	1 year 7 months	3 years	–	–
Walking	–	4 years 5 months	3 years 4 months	–	7 years 2 months	–	–	–
Characteristic face	+	+	+	+	+	+	+	+
HSCR	+	+	+	+	−‡	+	+	NE‡
Epilepsy	+	+	+§	–	+	+	–	–
Febrile convulsion	+	–	+	–	–	–	+	–
Heart disease	PDA, PH	–	–	–	Dextrocardia	AS	TOF	DORV, PA, AVSD, hypoLV, PDA, RAA
Brain MRI/CT	HCC (moderate), cortical atrophy	WNL	WNL	HCC (mild), cavum septi pellucidi	WNL	WNL	ACC, cortical atrophy	WNL
Other findings	Scoliosis		HPS		Pes planovalgus, ossifying fibroma	Hip joint contracture	Broad nasal bridge	Cleft hard palate
Present status								
Height	104.3 cm (−4.1 SD)	159.4 cm (+0.3 SD)	91.0 cm (−0.4 SD)	86.7 cm (−1.8 SD)	142.5 cm (−4.7 SD)	149.5 cm (−3.6 SD)	77.5 cm (−4.0 SD)	65.0 cm (−4.4 SD)
Body weight	15.9 kg (−2.3 SD)	43.5 kg (−1.3 SD)	12.6 kg (−0.5 SD)	12.0 kg (−1.1 SD)	28.0 kg (−3.3 SD)	38.0 kg (−2.3 SD)	8.6 kg (−3.0 SD)	6.3 kg (−3.0 SD)
HC	47.0 cm (−3.6 SD)	50.5 cm (−3.0 SD)	45.8 cm (−2.5 SD)	47.0 cm (−2.3 SD)	50.5 cm (−3.6 SD)	49.8 cm (−4.4 SD)	38.2 cm (−8.6 SD)	39.0 cm (−5.6 SD)
Speech	No	A few words	No	No	No	No	No	No
Gait	With support	Steadily	Steadily	No	Steadily	With support	No	No

*See Wakamatsu *et al*,[11] Yamada *et al*,[14] and Nagaya *et al*[17]; †see Wakamatsu *et al* [11]; ‡showed severe constipation and abdominal distension in early infancy; §showed repeated vomiting attacks in addition to epileptic convulsions.
(+), Presence of clinical signs; (−), absence of clinical signs.
ACC, agenesis of corpus callosum; AS, aortic valvular stenosis; AVSD, atrioventricular septal defect; DORV, double outlet right ventricle; DQ, developmental quotient; HC, head circumference; HCC, hypoplasia of corpus callosum; HPS, hypertrophic pyloric stenosis; HSCR, Hirschsprung disease; hypoLV, hypoplastic left ventricle; NE, not examined; PA, pulmonary atresia; PDA, patent ductus arteriosus; PH, pulmonary hypertension; RAA, right aortic arch; S. vaginal, spontaneous vaginal; TOF, tetralogy of Fallot; WNL, within normal limits.

Moreover, PAS was first identified in a patient with a *ZFHX1B* mutation. Despite this wide range of clinical features, all of the patients had heterozygous nonsense and frameshift mutations only in *ZFHX1B*. Zfhx1b (popularly known as Sip1) was recently isolated from mouse embryo using a yeast two-hybrid system as a Smad-interacting protein. It is a member of the vertebrate ZFHX1 family, and contains a Smad-binding domain, a homeobox-like domain, a transcriptional corepressor CtBP (carboxyl-terminal binding protein) interacting domain, and two separate clusters of zinc fingers (ZF) at its amino and carboxy terminals. Each ZF cluster binds independently, as a DNA-binding transcriptional repressor, to the promoter regions of genes like *CDH1* and *Cdh1*, encoding human and mouse E-cadherin.[24 25] A recent study demonstrated that full-length ZFHX1B can repress *CDH1* in a CtBP-independent manner.[26] This suggests that the CtBP interacting domain located at amino acid numbers 757–863 in ZFHX1B may have a different function than that of the ZF domains. Because all nonsense and frameshift mutations identified in patients are located on the amino-terminal side of the CtBP interacting domain, the prematurely truncated ZFHX1B in patients likely lack the CtBP-binding and ZF-mediated transcriptional repression activities encoded in the carboxy end of the protein. Thus, if the prematurely truncated protein is stable in certain patients, it is important to analyze its function in association with the clinical features of the disease. Defects in homozygous *Zfhx1b*-null mice show a more severe phenotype, such as a failure of neural tube closure and delaminating arrest of cranial neural crest cells,[27] demonstrating that *ZFHX1B* is essential for the normal development of brain and neural-crest precursors as well as midline structures in mammals.

Six patients with deletions located around 2q22, which included some of 2q23 in S1, S19, and S27, and encompassed

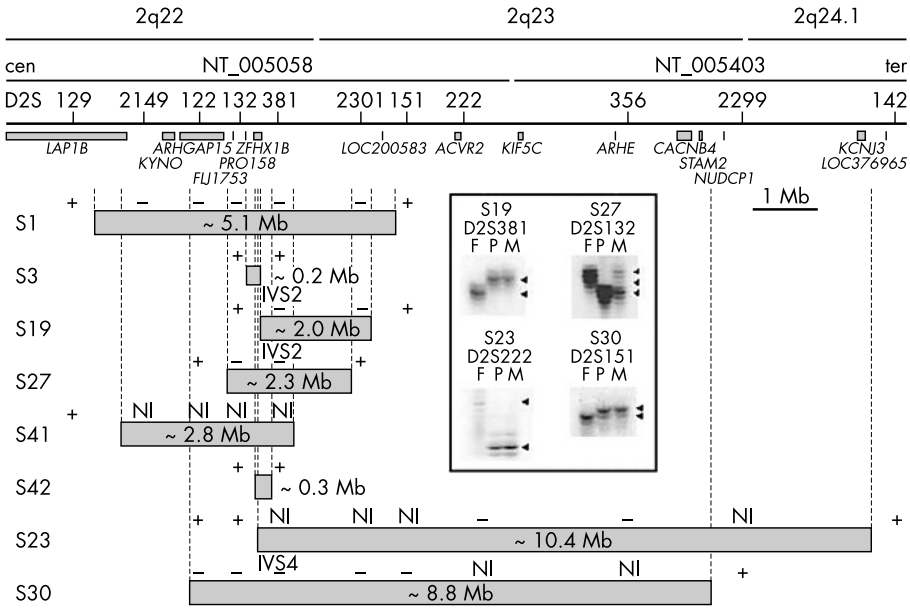

Figure 3 Schematic representation of the deletions in eight patients determined with microsatellite markers and multiplex PCR. Deletions from the patients are located at 2q22–q24.1. Microsatellite analysis of four patients (S19, S23, S27, and S30) indicates that the deletions occurred in the paternal allele. (+), located in both alleles; (−), in one allele; NI, not informative. F, P, and M indicate the patient's father, patient, and patient's mother, respectively.

part or all of *ZFHX1B* in all cases, demonstrated mental retardation, microcephaly, and hypertelorism in common. However, HSCR, epilepsy, congenital heart diseases, and abnormality of the corpus callosum were also observed in five, five, three, and two patients, respectively. Clearly, a wide variety of phenotypes is associated with deletions in these patients. These observations indicate that the clinical features in patients with deletions around 2q22 that encompass part or all of *ZFHX1B* are quite similar to those in cases with nonsense or frameshift mutations in *ZFHX1B*. This is consistent with cases reported recently.[28] Therefore, our data suggest that screening for deletions of *ZFHX1B* is warranted in patients presenting with the Mowat-Wilson phenotype, characterized by isolated cases, severe mental retardation, characteristic facial features, and microcephaly, even in the absence of a *ZFHX1B* mutation. S1 was previously considered to have more severe delayed psychomotor development than the three patients with nonsense and frameshift mutations, due to findings in S1 including DQ and brain atrophy.[11 17] Now, her DQ is 7 and she is able to walk with support. Moreover, a recent FISH study showed that the chromosomal breaking point at 13q22 in S1 was within two BAC clones of RP11-452B18 and RP11-115N13 (Ono *et al*, unpublished data). The interval spanned by these two BAC clones is about 1.1 Mb, and currently no deletions have been identified in this region. The differences between the clinical features caused by the 5.1 Mb deletion around 2q22 in S1 and those seen in other patients with mutations (table 1) or deletions up to 3 Mb (table 2) in *ZFHX1B* do not seem nearly as great as they did in the past. Thus, her clinical features are likely to be just phenotypic variations of the disease. Indeed, her clinical features as well as clinical course are quite distinguishable from those of S23 and S30, who had significantly delayed psychomotor development in association with large deletions at 2q22–q24.1. S30 also had a cleft of the hard palate and complicated congenital heart diseases such as DORV and hypoLV as additional features. These clinical features have not been reported in patients associated with *ZFHX1B* mutations and deletions, even though a cleft soft palate has been noted in cases with *ZFHX1B* mutations.[19] Moreover, a patient with an 11 Mb deletion reported by Zweier *et al*[28] presented with the remarkable clinical features of early seizures with a lethal course and hypoplasia of the big toes. Thus, the genes located at the region shared in the deletion interval at 2q23 in these three patients, such as *ARHE*, which encodes RhoE and functions in integrin-based focal adhesions and actin polymerization,[29] are candidates to affect brain development, midline structures of the face, and formation of the heart and extremities. However, these findings are not observed in another patient with a large deletion[30] including 2q23. Detailed clinical and molecular analyses of a number of patients with deletions at 2q23 that do not include *ZFHX1B* are important to consolidate our hypotheses concerning the role of the 2q23 deletion in this clinical phenotype.

We have also clarified the nucleotide sequence around the breakpoints of deletions in five patients. In a further two cases, S3 and S41, the breakpoints could be limited within a 1 kb region by repeating the multiplex PCR method, although we did not unequivocally determine the breakpoint sequences. The results suggest that the deletion mechanisms at 2q22–q24.1 are not simple, and that complexity might be associated, for example, with rearrangement of portions of genomic DNA. It should be noted that deletions in five cases (S19, S23, S27, S30, and S42) were not caused by mechanisms featuring inappropriate recombination between homologous sequences. The findings for all patients, including previously reported cases[15 28] examined thus far, have paternal deletions, also suggesting that chromosomal rearrangements occurred non-specifically around chromosome 2q22 during spermatogenesis. However, more detailed analyses of many patients are required to confirm this conclusion.

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