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The Spectrum of *ZEB2* Mutations Causing the Mowat–Wilson Syndrome in Japanese Populations

Yasukazu Yamada,[1] Noriko Nomura,[1] Kenichiro Yamada,[1] Mari Matsuo,[2] Yuka Suzuki,[3] Kiyoko Sameshima,[4] Reiko Kimura,[1] Yuto Yamamoto,[5] Daisuke Fukushi,[1] Yayoi Fukuhara,[1] Naoko Ishihara,[6] Eriko Nishi,[7] George Imataka,[8] Hiroshi Suzumura,[8] Shin-Ichiro Hamano,[9] Kenji Shimizu,[10] Mie Iwakoshi,[11] Kazunori Ohama,[12] Akira Ohta,[13] Hiroyuki Wakamoto,[14] Mitsuharu Kajita,[15] Kiyokuni Miura,[16] Kenji Yokochi,[17] Kenjiro Kosaki,[18] Tatsuo Kuroda,[19] Rika Kosaki,[20] Yoko Hiraki,[21] Kayoko Saito,[2] Seiji Mizuno,[7] Kenji Kurosawa,[22] Nobuhiko Okamoto,[5] and Nobuaki Wakamatsu[1]*

[1]Department of Genetics, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi, Japan

[2]Institute of Medical Genetics, Tokyo Women's Medical University, Shinjuku, Tokyo, Japan

[3]Department of Community Emergency Medicine, Graduate School of Medicine Ehime University, Tohon, Ehime, Japan

[4]Department of Medical Genetics, Gunma Children's Medical Center, Shibukawa, Gunma, Japan

[5]Department of Medical Genetics, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan

[6]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

[7]Department of Pediatrics, Central Hospital, Aichi Human Service Center, Kasugai, Aichi, Japan

[8]Department of Pediatrics, Dokkyo Medical University School of Medicine, Shimotsuga, Tochigi, Japan

[9]Division of Neurology, Saitama Children's Medical Center, Saitama, Saitama, Japan

[10]Division of Medical Genetics, Saitama Children's Medical Center, Saitama, Saitama, Japan

[11]Department of Healthcare Science, Kobe-Tokiwa University, Kobe, Hyogo, Japan

[12]Department of Pediatric Surgery, Ishikawa Prefectural Central Hospital, Kanazawa, Ishikawa, Japan

[13]Department of Pediatrics, Shikoku Medical Center for Children and Adults, Zentsuji, Kagawa, Japan

[14]Department of Pediatrics, Ehime Rehabilitation Center for Children, Tohon, Ehime, Japan

[15]Department of Pediatrics, Toyota Kosei Hospital, Toyota, Aichi, Japan

[16]Division of Developmental Disability Medicine, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

[17]Department of Pediatric Neurology, Seirei-Mikatahara General Hospital, Hamamatsu, Shizuoka, Japan

[18]Center for Medical Genetics, Keio University School of Medicine, Shinjuku, Tokyo, Japan

[19]Department of Pediatric Surgery, Keio University School of Medicine, Shinjuku, Tokyo, Japan

[20]Division of Medical Genetics, National Center for Child Health and Development, Setagaya, Tokyo, Japan

[21]Hiroshima Municipal Center for Child Health and Development, Hiroshima, Hiroshima, Japan

[22]Division of Medical Genetics, Kanagawa Children's Medical Center, Yokohama, Kanagawa, Japan

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Mowat–Wilson syndrome (MWS) is a multiple congenital anomaly syndrome characterized by moderate or severe intellectual disability, a characteristic facial appearance, microcephaly, epilepsy, agenesis or hypoplasia of the corpus callosum, congenital heart defects, Hirschsprung disease, and urogenital/renal anomalies. It is caused by de novo heterozygous loss of function mutations including nonsense mutations, frameshift mutations, and deletions in *ZEB2* at 2q22. *ZEB2* encodes the zinc finger E-box binding homeobox 2 protein consisting of 1,214 amino acids. Herein, we report 13 nonsense and 27 frameshift mutations from

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*Correspondence to:
Nobuaki Wakamatsu, M.D., Ph.D., Department of Genetics, Institute for Developmental Research, Aichi Human Service Center, 713-8 Kamiya-cho, Kasugai, Aichi 480-0392, Japan.
E-mail: nwaka@inst-hsc.jp

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40 newly identified MWS patients in Japan. Although the clinical findings of all the Japanese MWS patients with nonsense and frameshift mutations were quite similar to the previous review reports of MWS caused by nonsense mutations, frameshift mutations and deletions of *ZEB2*, the frequencies of microcephaly, Hirschsprung disease, and urogenital/renal anomalies were small. Patients harbored mutations spanning the region between the amino acids 55 and 1,204 in wild-type ZEB2. There was no obvious genotype–phenotype correlation among the patients. A transfection study demonstrated that the cellular level of the longest form of the mutant ZEB2 protein harboring the p.D1204Rfs*29 mutation was remarkably low. The results showed that the 3′-end frameshift mutation of *ZEB2* causes MWS due to ZEB2 instability.

Key words: Mowat–Wilson syndrome; frameshift mutation; nonsense mutation; ZEB2

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INTRODUCTION

Mowat et al. [1998] described six patients with severe intellectual disability, a distinct facial appearance, microcephaly, short stature, and Hirschsprung disease as a new syndrome. The authors also suggested that the disease locus was located at 2q22-23, because their only patient and a previously reported similar patient [Lurie et al., 1994] had deletions at 2q21-23 and 2q22-23, respectively. The determination of the chromosomal translocation breakpoint from two patients harboring the 2q22 translocation led to the identification of the zinc finger E-box binding homeobox 2 gene (*ZEB2*, also known as *ZFHX1B* and *SIP1*) as the disease gene [Cacheux et al., 2001; Wakamatsu et al., 2001]. Mowat–Wilson syndrome (MWS: OMIM#235730) was established as a distinct and recognizable syndrome; in particular, the characteristic facial appearance, which includes frontal bossing, eyebrows with medially flaring, hypertelorism, telecanthus, a broad nasal bridge, prominent columella, a prominent chin, and anomalies of ears, was associated with loss of function mutations (e.g., nonsense mutations, frameshift mutations, and deletions) in one allele of *ZEB2* [Zweier et al., 2002; Mowat et al., 2003]. Numerous reports (approximately 200) of *ZEB2* mutations in MWS [for clinical summaries or a review, see Zweier et al., 2005; Dastot-Le Moal et al., 2007; Garavelli et al., 2009] have been described. There is no obvious genotype–phenotype correlation in the MWS patients showing loss of function *ZEB2* mutations except for two patients with large deletions (>10 Mb) at the 2q22-24 locus, who presented with quite severe conditions and different original cases [Zweier et al., 2003; Ishihara et al., 2004]. MWS is caused by de novo mutations in one allele of *ZEB2*. The parents of MWS patients are usually healthy, and genetic abnormalities including apparent somatic mosaicism have not been reported. However, four families with MWS in siblings have been reported to be likely caused by germ-line mosaicism [McGaughran et al., 2005; Zweier et al., 2005; Cecconi et al., 2008; Ohtsuka et al., 2008].

ZEB2 is a member of the family of the two-handed zinc finger/homeodomain proteins containing an SMAD-binding domain (p.437–487), a homeodomain-like sequence (p.651–700), and two separate clusters of zinc fingers: an N-terminal domain (p.213–304) and a C-terminal domain (p.1001–1076) [Verschueren et al., 1999]. ZEB2 also possesses domains that interact with the nucleosome remodeling and histone deacetylation (NuRD) complex (p.14–17, 20) [Verstappen et al., 2008] and the transcriptional co-repressor C-terminal binding protein (p.757–863) [Postigo et al., 2003]. Recently, several Zeb2 functions in neuronal development and maturation have been identified by analyzing conditional knockout mice. Firstly, Zeb2 regulates the production of signals from post-mitotic cells back to the germinal zone to ensure the sequential generation of appropriate numbers of different neurons and glial cells throughout corticogenesis [Seuntjens et al., 2009]. Secondly, Zeb2 is essential for central nervous system myelination through the modulation of two distinct regulatory pathways (i.e., BMP-Smad and Wnt-β-catenin pathways) [Weng et al., 2012]. Thirdly, Zeb2 promotes a fate switch between cortical and striatal interneuron lineages through the repression of Nkx2-1 during neuronal migration from the medial ganglionic eminence [McKinsey et al., 2013].

Here, we report on nonsense and frameshift *ZEB2* mutations and the clinical features of 40 newly identified MWS patients in Japan. One patient carries the frameshift mutation of p.D1204Rfs*29 at the C-terminal of *ZEB2*; the mutant ZEB2 shares 99% (1,203/1,214) of its amino acids with the wild-type protein. We analyze the C-terminal mutant of ZEB2 and discuss the pathogenesis of the disease.

MATERIALS AND METHODS

Clinical Studies of MWS

Written informed consent was obtained from all the participants of this study. The experiments were conducted after approval by the Institutional Review Board at the Institute for Developmental Research, Aichi Human Service Center. The patients participating in this study were labeled S-001–S-131, except for five patients (K-01, K-02, O-01, P-1, P-2), whose *ZEB2* analysis was separately performed. S-073 (a-c) are sibling cases. The clinical and molecular analysis of *ZEB2* from S-001–S-042, S-073 (a-c), and P-1 and P-2 have been published elsewhere [Wakamatsu et al., 2001; Yamada

et al., 2001; Ishihara et al., 2004; Sasongko et al., 2007; Ohtsuka et al., 2008]. We performed the genetic analysis of the *ZEB2* in Japanese patients with a potential clinical diagnosis of MWS and presented the confirmed MWS cases based on *ZEB2* analysis at the corresponding meetings of physicians in charge by supporting the Research on Measures for Intractable Diseases sponsored by the Ministry of Health Labor and Welfare in Japan. The prevalence of MWS was determined by epidemiological survey of the patients from the hospitals and medical centers for pediatric rehabilitation at the Aichi and Kanagawa prefectures.

DNA Sequencing

Genomic DNA was isolated from the peripheral blood of the patients with a possible clinical diagnosis of MWS and the mutations in *ZEB2* were evaluated as previously described [Yamada et al., 2001]. Briefly, nine PCR products encompassing all nine coding exons (exons 2–10) including intron/exon boundaries were amplified and sequenced directly. To confirm the mutations detected in one allele of the patients, the PCR products were subcloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. The nucleotide sequence of the DNA fragment was determined using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, CA), with the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).

Construction of the Wild-Type and Mutant ZEB2 Expression Vectors

Wild-type ZEB2 cDNA was amplified from the first-strand cDNA prepared from HEK293 cells using the specific primer pair S1-A1 (S1, exon 1: 5′-cgctgaattcaatgaagcagccgatcatg-3′; A1, exon 10: 5′-aatgctctagattattacatgccatc-3′). An *Eco*RI recognition site (gaattc) or an *Xba*I recognition site (tctaga) was introduced into S1 or A1, respectively. After confirming the nucleotide sequences, the *Eco*RI/*Xba*I fragment of the wild-type ZEB2 cDNA was subcloned into the *Eco*RI/*Xba*I site of a mammalian expression vector, p3xFLAG-CMV (Sigma–Aldrich, St. Louis, MO) (pFLAG-ZEB2). To generate the D1204Rfs*29 mutant of ZEB2, the 3′ portion of the *ZEB2* was amplified with the primer pair S2-A2 (S2, exon 10: 5′-cgggcttacttg-cagagcat-3′; A2, exon 10: 5′-catgaacagcttaactctagagtgttttc-3′) using the genomic DNA prepared from the patient's peripheral blood cells. An *Xba*I recognition site was introduced into A2. A 189-bp piece of the *Bam*HI/*Xba*I fragment of pFLAG-ZEB2 was exchanged with a 184-bp piece of *Bam*HI/*Xba*I-digested PCR fragment (pFLAG-ZEB2-D1204Rfs*29). Similarly, the ZEB2 expression vectors (pFLAG-ZEB2-D1204X and pFLAG-ZEB2-M1210X) containing premature termination codons at the 3′-end were generated by in vitro mutagenesis. The nucleotide sequences of all the constructed ZEB2 expression vectors were verified by sequencing.

Expression Study of Wild-Type and Mutant ZEB2 Proteins in HEK293 Cells

Each ZEB2 expression vector (4 μg; p3xFLAG-CMV, pFLAG-ZEB2, pFLAG-ZEB2-D1204Rfs, pFLAG-ZEB2-D1204X, and pFLAG-ZEB2-M1210X) was cotransfected with 50 ng of pCMV-β-gal (an *Escherichia coli* β-galactosidase expression vector) into HEK293 cells in six-well dishes using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After a 24-h transfection, the cells from each well were harvested and replated in two wells of six-well dishes. After a 48-h transfection, the cells from one of the two wells were washed with PBS, solubilized with a lysis buffer containing 20 mM Tris–HCl (pH 7.5) and Protease Inhibitor Cocktail (1:1,000 dilution), and sonicated using SOMIFIER 250 (BRANSON, Danbury, CT). The FLAG tagged ZEB2 mRNA levels relative to mRNA of β-actin (ACTB) were analyzed by the multiplex PCR method [Ishihara et al., 2004]. Total RNA was extracted from HEK293 cells transfected with each of the ZEB2-expressing vectors using TRIzol Reagent (Invitrogen) and first-strand cDNAs were synthesized by reverse transcription of 4.5 μg of total RNA using First-Strand cDNA Synthesis Kit (GE Healthcare, Tokyo, Japan). Primer pairs were designed to amplify a 178-bp fragment (90-bp of FLAG and 88-bp of ZEB2) of FLAG tagged ZEB2 cDNA: S3 (sense primer for the FLAG sequence), 5′-aaccatggactacaaagacca-3′ and A3 (antisense primer for exons 1 and 2 of ZEB2), 5′-cattgtcatagtt-caccacgt-3′, and a 149-bp fragment of ACTB: S4, 5′-gacaggatgcaga-aggagat-3′ and A4, 5′-ctgcttgctgatccacatct-3′. Aliquots (equivalent to 0.1 μg of total RNA) of first-strand cDNA were amplified by PCR in a total volume of 20 μl, each containing 0.3 μM of the both primer pairs (S3-A3 and S4-A4), and 20 cycles were performed. PCR products were separated on 1.5% low melting point agarose gel electrophoresis. Western blotting was performed using an anti-FLAG M2 antibody (1:6,000 dilution; Sigma–Aldrich) following the same method as described elsewhere [Yamada et al., 2013]. Proteins were analyzed using ImageQuant LAS 4000 mini (GE Healthcare). The efficiency of the DNA transfection was verified by measuring the *E. coli* β-galactosidase activity using O-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.

RESULTS

The Prevalence of MWS

The epidemiological survey demonstrated that the prevalence of MWS at the Aichi and Kanagawa prefectures is 1:74,000 and 1:110,000, respectively. Thus, similar to the results of a previous report [Evans et al., 2012], the prevalence of MWS in Japan is approximately 1:90,000.

Identification of Nonsense and Frameshift Mutations in *ZEB2*

We have identified nonsense mutations in 13 new patients (Table I). The mutation p.R695X, which was previously reported in eight patients [Ishihara et al., 2004; Sasongko et al., 2007], was found in four new patients. In this study, the mutations p.R343X and p.R921X were newly detected in two patients, respectively. We have already presented a patient with p.R343X mutation [Ishihara et al., 2004], while p.R921X was previously reported in European patients [Zweier et al., 2005; Garavelli et al., 2009]. Five new mutations (i.e., p.Q271X, p.C312X, p.E609X, p.S800X, and p.S872X) have been identified in this study. In total, 13 kinds of nonsense mutations were found in 29 patients including three sibling cases (Table I). A total of

TABLE I. MWS Patients Associated With Nonsense *ZEB2* Mutations in the Japanese Population

No.	Case	Age	GD	EX	Mutation	Amino acid	ID	CFA	MC	CHD	S	HSCR	ACC/HCC	SP	Others	RF
1	S-073.a[a]	2 y 7 m	M	3	c.259G>T	p.E87X	++	+	−	−	+	−	−	−	−	[a]
2	S-073.b[a]	5 y 7 m	F	3	c.259G>T	p.E87X	++	+	−	−	+	−	−	−	−	[a]
3	S-073.c[a]	4 y 6 m	F	3	c.259G>T	p.E87X	++	+	−	−	+	−	ND	−	−	[a]
4	S-122	1 y 8 m	F	7	c.811C>T	p.Q271X	+	+	+	−	−	−	H	−	−	This study
5	S-031	7 y	M	7	c.904C>T	p.R302X	++	+	+	−	+	+	−	−	Cryptorchism	[b]
6	S-062	25 y	M	8	c.936C>A	p.C312X	++	+	+	ASD, PDA	+	+	A	B	Hypospadias, urinary disturbance, urethral stone	This study
7	S-014	4 y	M	8	c.1027C>T	p.R343X	++	+	+	ASD, PS	−	+	ND	−	Hypospadias	[b]
8	K-02	6 y	M	8	c.1027C>T	p.R343X	++	+	−	−	+	−, CO	−	−	Spinal bifida	This study
9	O-01	2 y	M	8	c.1027C>T	p.R343X	++	+	−	PDA	+	−, CO	H	−	Otitis media	This study
10	P-2[b]	8 y	F	8	c.1298C>T	p.Q433X	++	+	−	−	+	+	−	−	−	[c]
11	S-034	28 y	M	8	c.1489C>T	p.Q497X	++	+	−	−	+	−	ND	−	−	[b]
12	S-002	20 y	F	8	c.1645A>T	p.R549X	++	+	+	PDA	−	+	A	−	Exotropia	[d, b]
13	S-046	12 y 9 m	M	8	c.1825G>T	p.E609X	++	+	+	−	+	−, CO	H	−	−	This study
14	S-004	25 y	M	8	c.2083C>T	p.R695X	++	+	+	−	+	+	ND	−	HPS	[d, b]
15	S-006	28 y	M	8	c.2083C>T	p.R695X	++	+	+	−	+	−, CO	−	−	Exotropia	[b, e]
16	S-007	30 y	M	8	c.2083C>T	p.R695X	++	+	+	VSD	+	−, CO	−	−	Cryptorchism, esotropia	[b, e]
17	S-008	26 y	M	8	c.2083C>T	p.R695X	++	+	+	−	+	−, CO	−	−	−	[b, e]
18	S-021	6 y	M	8	c.2083C>T	p.R695X	++	+	+	PDA	+	+	ND	−	Hypospadias	[b]
19	S-032	2 y	M	8	c.2083C>T	p.R695X	++	+	−	PDA, VSD	+	+	H	−	Cryptorchism, hypospadias, HPS	[b]
20	S-038	3 y	F	8	c.2083C>T	p.R695X	++	+	+	PDA	+	+	−	−	Septum of vagina, exotropia	[b]
21	P-1[b]	5 y	F	8	c.2083C>T	p.R695X	++	+	+	PDA, VSD, AS, PS	+	−	−	−	Duplicated renal pelvis	[c]
22	S-097	14 y 8 m	F	8	c.2083C>T	p.R695X	++	+	−	−	−	−, CO	−	−	−	This study
23	S-112	10 y 9 m	M	8	c.2083C>T	p.R695X	++	+	+	PS	+	+	−	−	Hypospadias, otitis media, spleen hypoplasia	This study
24	S-118	3 y	M	8	c.2083C>T	p.R695X	++	+	−	−	−	−, CO	H	−	−	This study
25	S-120	2 y 11 m	M	8	c.2083C>T	p.R695X	++	+	+	ASD PS	+	−, CO	H	−	CWH, otitis media	This study
26	S-098	10 y	M	8	c.2399C>G	p.S800X	++	+	+	VSD PS	+	−	H	FW	Conduction deafness, exotropia	This study
27	S-054	8 y	F	8	c.2615C>G	p.S872X	++	+	+	−	+	−, CO	A	B	−	This study
28	S-101	6 y	M	8	c.2761C>T	p.R921X	++	+	+	VSD ASD	+	−	ND	B	Self-injury	This study
29	S-111	3 y 11 m	F	8	c.2761C>T	p.R921X	++	+	−	PDA	+	+, Short	−	−	−	This study

[a]The mutation was detected in three sibling patients [S-073.a–c] [a].
[b]P-1 and P-2 were reported as Patients 1 and 2, respectively [c].
GD, gender; EX, exon; ID, intellectual disability [++ severe, + moderate]; CFA, characteristic facial appearance; MC, microcephaly; CHD, congenital heart disease; S, seizures; HSCR, Hirschsprung disease; ACC/HCC, agenesis of the corpus callosum/hypoplasia of corpus callosum; SP, speech; RF, references; y, year; m, month; M, male; F, female; ND, not determined; ASD, atrial septal defect; PDA, patent ductus arteriosus; VSD, ventricular septal defect; AS, aortic stenosis; PS, pulmonary stenosis; CO, constipation; A, agenesis; H, hypoplasia; B, babbling; FW, few words; CWH, chordee without hypospadias; a, Ohtsuka et al. [2008]; b, Ishihara et al. [2004]; c, Sasongko et al. [2007]; d, Wakamatsu et al. [2001]; e, Yamada et al. [2001].

TABLE II. MWS Patients Associated With Frameshift *ZEB2* Mutations in the Japanese Population

No.	Case	Age	GD	EX	Mutation	Amino acid	ID	CFA	MC	CHD	S	HSCR	ACC/HCC	SP	Others	RF
1	S-108	7 y	M	3	c.[162_164]delC [CCC>CC]	p.P55Lfs*20	++	+	+	−	+	+	H	−	−	This study
2	S-128	5 y	M	3	c.[175_182]del5-bp [GAGACGAG>GAG]	p.T60Sfs*3	++	+	−	VSD	+	−	−	FW	Otitis media, hydronephrosis, self-injury	This study
3	S-010	6 y	F	3	c.[270_272]delG [GGG>GG]	p.G91Vfs*17	++	+	+	−	−	−	H	−	−	[b, e]
4	S-058	10 y	M	3	c.[311_312]dupA [AA>AA[A]]	p.A105Sfs*16	++	+	−	PDA	+	+	H	−	Hypospadias	This study
5	S-094	12 y 6 m	M	5	c.[459_460]delG [GG>G]	p.E154Rfs*58	++	+	+	PDA, AS, PS	−	+, Short	H	−	Bifid scrotum, blepharoptosis	This study
6	S-113	6 y	F	6	c.635_638dupCCTG [CCTG>CCTG[CCTG]]	p.P214Lfs*26	++	+	−	VSD	+	+	−	B	−	This study
7	S-115	5 y	F	6	c.647delG	p.C216Sfs*8	++	+	−	AS	−	+	H	−	−	This study
8	S-009	27 y	M	6	c.759_760dupCA [CA>CA [CA]]	p.Q255Pfs*8	++	+	+	−	+	−	−	−	−	[b, e]
9	S-047	15 y	M	7	c.[852_855]del2-bp [CACA>CA]	p.T285Rfs*9	++	+	+	ASD	+	+	H	−	Otitis media	This study
10	S-028	10 y	M	7	c.[855_858]del2-bp [AGAG>AG]	p.E286Vfs*8	++	+	+	PAS, PDA	+	−, CO	A	−	Cryptorchism	[b]
11	S131	1 y	M	7	c.[862_863]delG [GG>G]	p.G288Afs*10	ND	+	+	−	−	CO	A		Hypospadias	This study
12	S-015	5 y	M	8	c.1169ins382-bp [CAGGCCGGGE, 382-bp]	p.I390Tfs*41	++	+	+	−	+	−	H	−	−	[b]
13	S-076	11 y	F	8	c.[1169_1170]delT [TT>T]	p.T392Qfs*4	++	+	−	−	+	−	−	−	Exotropia	This study
14	S-005	25 y	M	8	c.[1174_1178]del4-bp [ACAGA>A]	p.T392Nfs*3	++	+	+	PDA	+	+	−	−	−	[d, b]
15	S-068	19 y	M	8	c.1176delG	p.E393Nfs*3	++	+	−	PDA, PS	+	+	−	−	HPS	This study
16	S-127	21 y	F	8	c.[1212_1213]delG [GG>G]	p.A405Lfs*12	++	+	−	PS	+	−, CO	−	FW	−	This study
17	S-103	6 y 2 m	F	8	c.[1268_1273]del4-bp [CCAGCC>CC]	p.S424Lfs*2	+	+	+	VSD	−	+	H	FW	AH	This study
18	S-050	18 y	M	8	c.1280_1286del7insACTGAG [GAGTTCA>ACTGAG]	p.G427Dfs*2	++	+	ND	−	ND	+, Long	ND	−	−	This study
19	S-102	8 y 10 m	F	8	c.[1334_1337]dupC [CCCC>CCCC[C]]	p.L447Ffs*9	++	+	+	ASD, PH, PS	+	−, CO	H	−	−	This study
20	S-024	11 y	F	8	c.1395_1408del14ins19 [GATTE,14-bp>CAAGE,19-bp]	p.Q465Hfs*9	++	+	+	PDA	+	+	−	−	−	[b]
21	S-090	8 y	F	8	c.1417delA	p.R473Gfs*14	++	+	+	−	+	−, CO	H	−	−	This study
22	K-01	8 y	F	8	c.1417delA	p.R473Gfs*14	++	+	+	−	+	−	−	−	Otitis media	This study
23	S-104	6 y 6 m	F	8	c.[1421_1426]dupA [AAAAAA>AAAAAA[A]]	p.M476Nfs*6	++	+	+	−	−	−, CO	−	−	Coloboma	This study
24	S-110	4 y 10 m	F	8	c.[1492_1493]delC [CC>C]	p.P498Lfs*18	++	+	−	PDA. T/F	+	−, CO	−	−	Duplicated renal pelvis	This study
25	S-066	12 y	F	8	c.[1534_1535]delG [GG>G]	p.G512Vfs*4	++	+	−	VSD	+	−, CO	−	FW	Hydronephrosis	This study
26	S-065	9 y	F	8	c.1822delG	p.E608Kfs*13	++	+	+	PDA	+	−, CO	−	−	−	This study
27	S-130	2 y 6 m	M	8	c.1966_1967delAT	p.M656Vfs*17	++	+	+	T/F	−	−, CO	−	−	HPS, high arched palate	This study
28	S-011	3 y	M	8	c.[2178_2180]delTT [TTT>T]	p.L727Ifs*28	++	+	+	−	+	−, CO	H	−	Exotropia	[b]

(*Continued*)

TABLE II. (*Continued*)

No.	Case	Age	GD	EX	Mutation	Amino acid	ID	CFA	MC	CHD	S	HSCR	ACC/HCC	SP	Others	RF
29	S-063	9 y	F	8	c.2254dupA [A>A[A]]	p.T752Nfs*4	++	+	+	T/F	+	+	A	B	Otitis media	This study
30	S-100	10 y 7 m	F	8	c. 2282delC	p.T761Kfs*26	++	+	–	PDA	+	–, CO	A	–	Otitis media	This study
31	S-106	12 y	F	8	c.[2349_2351]dupT [TT>TT[T]]	p.S784Ffs*11	++	+	+	–	+	–, CO	–	–	–	This study
32	S-049	23 y	M	8	c.2579delT	p.L860Rfs*3	++	+	+	–	+	+	H	–	Otitis media	This study
33	S-125	1 y 10 m	M	8	c.2740_2743dupCAGA [CAGAC>CAGA[CAGA]C]	p.S916Dfs*34	++	+	–	–	–	–	–	–	Cryptorchidism, hydronephrosis, intermittent exotropia	This study
34	S-121	1 y 10 m	M	10	c.[3608_3614]del5-bp [CAGACCA>CA]	p.D1204Rfs*29	++	+	–	PDA, VSD	+	–	H	–	Hypospadias	This study

EX, exon; ID, intellectual disability [++ severe, + moderate]; ND, not determined; CFA, characteristic facial appearance; MC, microcephaly; CHD, congenital heart disease; S, seizures; HSCR, Hirschsprung disease; ACC/HCC, agenesis of the corpus callosum/hypoplasia of corpus callosum; SP, speech; RF, references; y, year; m, month; M, male; F, female; ASD, atrial septal defect; PDA, patent ductus arteriosus; PAS, pulmonary artery sling; VSD, ventricular septal defect; AS, aortic stenosis; PH, pulmonary hypertension; PS, pulmonary stenosis; T/F, tetralogy of Fallot; CO, constipation; A, agenesis; H, hypoplasia; B, babbling; FW, few words; HPS, hypertrophic pyloric stenosis; AH, astigmatismus hypermetropicus; b, Ishihara et al. [2004]; d, Wakamatsu et al. [2001]; e, Yamada et al. [2001].

33 frameshift mutations from Japanese patients caused by small deletions, duplications, insertions, or other phenomena are summarized in Table II. In this study, 26 frameshift mutations were newly identified in 27 patients, and all the mutations were scattered between P55 and D1204. Only c.1417delA resulting in p.R473Hfs*14 was detected in two different patients. The mutations c.(852_855)del2-bp, c.(1421_1426)dupA, and c.2254dupA have been reported in European patients [Cacheux et al., 2001; Zweier et al., 2005; Garavelli et al., 2009]. Among the reported cases, the c.(3608_3614)del5-bp resulting in p.D1204Rfs*29 mutation is the closest to the C-terminal end.

Clinical Features in MWS Patients Associated With Nonsense and Frameshift Mutations

The clinical features of newly identified and previously reported Japanese patients with MWS harboring nonsense and frameshift mutations are summarized in Table III. All the patients showed severe to moderate intellectual disability and a characteristic facial appearance. It is noted that the sex ratio and most of the clinical features of these cases are quite similar to those in previous reports [Garavelli et al., 2009]. A detailed comparison of the results of this study with those from an earlier report showed that male/female ratio was 1.33 and 1.25, seizure frequencies were 78% and 74%, abnormalities of the corpus callosum were seen in 44% vs. 46%, and congenital heart disease was seen 54% and 54%, respectively. Five patients (S-066, S-098, S-103, S-127, and S-128) could speak a few words, and five patients (S-066, S-068, S-098, S-127, and S-128) could point at objects (e.g., food) kept out of reach. Characteristic facial appearance of seven patients is shown in Figure 1.

Instability of the ZEB2-D1204Rfs*29 Protein

The ZEB2-D1204Rfs*29 allele in S-121 encodes the longest 1,203 amino acid stretch from wild-type ZEB2 and an insertion of an additional 28 amino acids caused by a frameshift mutation at the C-terminus (total 1,231 amino acids). The molecular mass of FLAG-tagged wild-type ZEB2 and ZEB2-D1204Rfs*29 were calculated to be 139.7 and 141.9 kDa, respectively. To characterize the mutant ZEB2, we examined the mRNA levels of the transiently expressed wild-type and mutant ZEB2 by multiplex PCR, but no marked differences were observed (Fig. 2B). Next, we performed Western blotting in the cells. The results demonstrated that the ZEB2 protein level in cells harboring the p.D1204Rfs*29 mutation is remarkably decreased compared to that of the wild-type-expressing cells. In contrast, the ZEB2 protein levels in cells harboring the p.D1204X or p.M1210X mutants were not decreased. Moreover, the expression of ZEB2-D1204Rfs*29 was approximately 20% that of the wild-type (Fig. 2B) and the difference in the molecular mass of ZEB2-D1204Rfs*29 and wild-type ZEB2 was found to be more than 10 kDa (Fig. 2C). This is larger than the calculated MW difference of the two proteins, which is 2.2 kDa.

DISCUSSION

To date, more and more pediatricians, pediatric neurologists, pediatric surgeons, human geneticists, and genetic counselors in

TABLE III. Clinical Features of MWS Patients Associated With Nonsense and Frameshift Mutations

	Nonsense mutations, Table I [A]		Frameshift mutations, Table II [B]		
	This study (n = 13)	Total (n = 29)	This study (n = 27)	Total (n = 34)	[A] + [B] (n = 63)
Male/female	9/4	19/10	12/15	17/17	36/27
Intellectual disability	All	All	All	All	All
Microcephaly	8/13 (62%)	18/29 (62%)	14/27 (52%)	21/34 (62%)	39/63 (62%)
Seizures	10/13 (77%)	24/29 (83%)	19/27 (70%)	25/34 (74%)	49/63 (78%)
Hypoplasia or agenesis of the corpus callosum	9/13 (69%)	11/29 (38%)	13/27 (48%)	17/34 (50%)	28/63 (44%)
Congenital heart disease	7/13 (54%)	14/29 (48%)	17/27 (63%)	20/34 (59%)	34/63 (54%)
Hirschsprung disease	3/13 (23%)	11/29 (38%)	11/27 (41%)	13/34 (38%)	24/63 (38%)
Constipation	7/13 (54%)	10/29 (35%)	11/27 (41%)	13/34 (38%)	23/63 (37%)
Urogenital/renal anomalies	3/13 (23%)	10/29 (35%)	8/27 (30%)	9/34 (26%)	19/63 (30%)

FIG. 1. Facial appearance of MWS patients. A: S-97 (12-year-2-month-old female). B: S-98 (4-year-10-month-old male). C: S-100 (7-year-2-month-old female). D: S-110 (1-year-8-month-old male). E: S-8 (36-year-old male). F: S-94 (10-year-5-month-old male). G: S-111 (1-year-10-month-old female). The patients have eyebrows with medially flaring and sparse in the lateral, large and deep-set eyes, telecanthus, broad nasal bridge, depressed nasal bridge, prominent and triangular chin, and uplifted ear lobes. Smiling face (A, B, E, F), thin chestnut hair (B–D, G), frontal bossing (B–D, G), hypertelorism (A–E), round nasal tip with a prominent columella and a short philtrum (A–F) and posteriorly rotated ears (A, B, D) are also noted. S94 (F) has right ptosis.

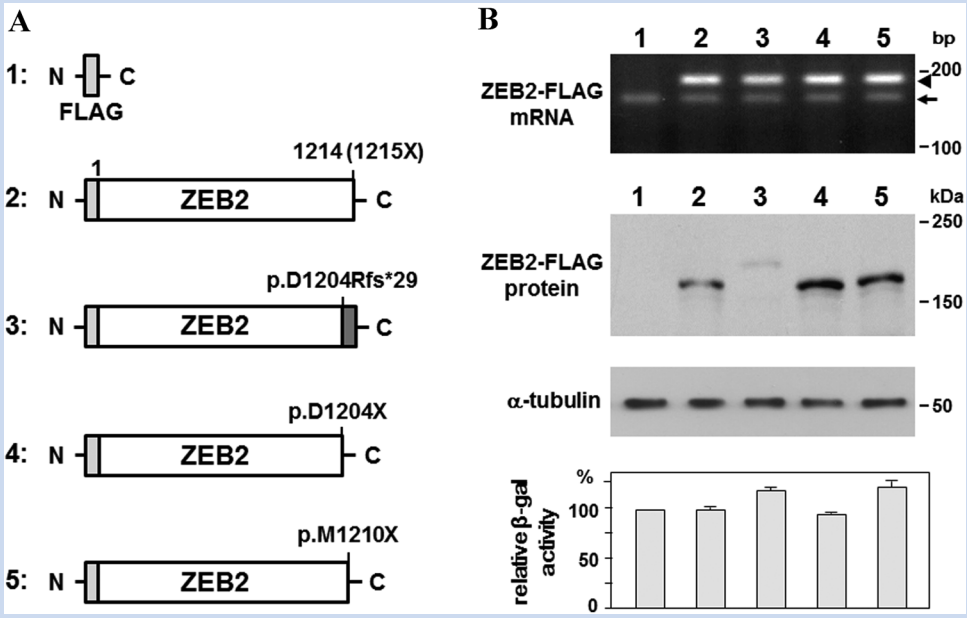

FIG. 2. Expression of wild-type and mutant ZEB2 proteins in HEK293 cells. A: A schematic illustration of FLAG-tagged ZEB2 mutants. B: First (topmost) panel. Multiplex PCR analysis of the FLAG tagged ZEB2 mRNA levels relative to ACTB in HEK293 cells transfected with each of the ZEB2-expressing vectors. Arrow and arrowhead indicated the PCR products of ACTB and FLAG tagged ZEB2, respectively. Second and third panels: Western blot analysis of HEK293 cells transfected with each of the ZEB2-expressing vectors using antibodies specific for FLAG and α-tubulin. Fourth panel: relative *E. coli* β-galactosidase (β-gal) activities in each transfected group of HEK293 cells. The vertical bars indicate the standard error of the mean for three experiments. Lane 1, pFLAG vector; lane 2, wild-type pFLAG-ZEB2; lane 3, pFLAG-ZEB2-D1204Rfs*29; lane 4, pFLAG-ZEB2-D1204X; lane 5, pFLAG-ZEB2-M1210X.

Japan recognize MWS based on the characteristic facial appearance and moderate or severe intellectual disability, similar to that seen in Down syndrome. There is no obvious genotype–phenotype correlation in the MWS patients except for large deletions (>10 Mb) at the 2q22-24 locus [Zweier et al., 2003; Ishihara et al., 2004]; yet we speculate that clinical features of MWS may become rather unclear when we include the deletion types harboring wide clinical symptoms. Thus, we have focused on the clinical features of MWS caused by nonsense and frameshift mutations in *ZEB2* in this study. We have identified 13 nonsense and 27 frameshift *ZEB2* mutations in 40 newly identified MWS patients. Thus, the overall number of nonsense and frameshift mutations of the so far identified Japanese MWS patients are 29 and 34, respectively, including those from previous studies [Yamada et al., 2001; Ishihara et al., 2004; Sasongko et al., 2007; Ohtsuka et al., 2008]. All the patients have the characteristic facial appearance, and moderate or severe intellectual disability. Compared to previous reports of the clinical summary of MWS including deletion cases [Dastot-Le Moal et al., 2007], the frequencies of patients showing seizures, abnormalities of corpus callosum, or congenital heart disease are quite similar; however, the frequencies of microcephaly (62% vs. 80%), Hirschsprung disease (38% vs. 54%), and urogenital/renal anomalies (30% vs. 52%) are lower than those previously reported (Table III) [Garavelli et al., 2009]. We observed that microcephaly is evident at a later age in some patients. For example: (1) S-090, occipitofrontal circumference (OFC): −1.0 standard deviation (SD) (at birth) and −2.5 SD (3 years old), (2) S-106, OFC: 0 SD (at birth) and −2 SD (9 years old) with failure to thrive. Thus, microcephaly may not be characteristic of the younger patients (~6 years old) in this study. Further case studies are necessary to investigate whether nonsense and frameshift mutations in *ZEB2* are responsible for the relatively lower number of Hirschsprung disease and urogenital/renal anomalies in this study. In addition, the urethral stones (S-062), spleen hypoplasia (S-112), chordee without hypospadias (S-120), duplicated renal pelvis (S-110 and P-1), and spinal bifida (K-02) identified in this study are rare in MWS. Further case studies are also necessary to establish whether these are symptoms associated with MWS. We found that two patients (S-101 and S-128) have self-injuries, in accordance with a recent study showing that MWS was associated with significant levels of behavioral and emotional problems [Evans et al., 2012].

In this study, using transient transfection, we demonstrated that the ZEB2-D1204Rfs*29 protein has a larger mass, but its protein level was remarkably decreased without a remarkable change in the mRNA level when compared to that of the wild-type ZEB2. Thus, MWS was caused by the ZEB2 protein instability in S-121. The D1204Rfs*29 mutation generates a new terminal codon. Consequently, the 11 C-terminal amino acid sequence (DHEEDNMEDGM) encoded by wild-type ZEB2 was replaced by a 28-amino acid sequence (RGRQYGRWHVNYCILSFHFFFPVVLLPA) by the frameshift mutation. Comparing the two C-terminal peptides, 6 (underlined) out of 11 amino acids were negatively charged

amino acids, while 15 (double underlined) out of 28 amino acids were hydrophobic amino acids. This remarkably different amino acid composition suggests that conformation of the C-termini of the two ZEB2 proteins would differ. Secondary structure analysis of the C-terminus of the wild-type and D1204Rfs*29 proteins using "Jpred consensus method for protein secondary structure prediction server" indicated a dramatically increased potential for alpha helix formation with strong hydrophobicity in the C-terminal peptide of ZEB2-D1204Rfs*29 [Cole et al., 2008]. This could affect the post-translational modifications of mutant ZEB2, including processing, phosphorylation, or glycosylation as well as protein stability, which could explain the Western blot findings. A different C-terminal frameshift mutation in ZEB2 has been reported in MWS where the c.3567-3568insCC frameshift mutation (causing a 2-bp insertion) produces a longer ZEB2 (p.M1190Pfs*50) than ZEB2-D1204Rfs*29; however, the characterization of the mutant ZEB2 has not been performed. The finding that nonsense mutations (1204X and 1210X) at the C-terminus of ZEB2 do not affect the protein stability suggests that these mutations may not cause typical MWS. To confirm these hypotheses, C-terminal analysis of the mutant ZEB2, case studies of MWS with C-terminus mutations, or single nucleotide polymorphism analyses of normal populations are necessary.

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