

FULL-LENGTH ORIGINAL RESEARCH

CASK aberrations in male patients with Ohtahara syndrome and cerebellar hypoplasia

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SUMMARY

Purpose: Ohtahara syndrome (OS) is one of the most severe and earliest forms of epilepsy. *STXBPI* and *ARX* mutations have been reported in patients with OS. In this study, we aimed to identify new genes involved in OS by copy number analysis and whole exome sequencing.

Methods: Copy number analysis and whole exome sequencing were performed in 34 and 12 patients with OS, respectively. Fluorescence in situ hybridization, quantitative polymerase chain reaction (PCR), and breakpoint-specific and reverse-transcriptase PCR analyses were performed to characterize a deletion. Immunoblotting using lymphoblastoid cells was done to examine expression of CASK protein.

Key Findings: Genomic microarray analysis revealed a 111-kb deletion involving exon 2 of *CASK* at Xp11.4 in a male patient. The deletion was inherited from his mother, who was somatic mosaic for the deletion. Sequencing of the mutant transcript expressed in lymphoblastoid cell

lines derived from the patient confirmed the deletion of exon 2 in the mutant transcript with a premature stop codon. Whole exome sequencing identified another male patient who was harboring a c.1A>G mutation in *CASK*, which occurred de novo. Both patients showed severe cerebellar hypoplasia along with other congenital anomalies such as micrognathia, a high arched palate, and finger anomalies. No CASK protein was detected by immunoblotting in lymphoblastoid cells derived from two patients.

Significance: The detected mutations are highly likely to cause the loss of function of the CASK protein in male individuals. CASK mutations have been reported in patients with intellectual disability with microcephaly and pontocerebellar hypoplasia or congenital nystagmus, and those with FG syndrome. Our data expand the clinical spectrum of CASK mutations to include OS with cerebellar hypoplasia and congenital anomalies at the most severe end.

KEY WORDS: CASK, Ohtahara syndrome, Male, Cerebellar hypoplasia.

Ohtahara syndrome (OS), also known as early infantile epileptic encephalopathy with suppression-burst, is one of the most severe and earliest forms of epilepsy (Ohtahara et al., 1976). It is characterized by early onset of seizures, typically frequent epileptic spasms, seizure intractability, characteristic suppression-burst patterns on electroencephalography (EEG), and poor outcome with severe psychomotor retardation (Djukic et al., 2006; Ohtahara & Yamatogi, 2006). Brain malformations such as cerebral dysgenesis, hemimegalencephaly, Aicardi syndrome, and porencephaly

are often associated with OS (Yamatogi & Ohtahara, 2002). However, mutations of the *ARX* and *STXBPI* gene have been reported in individuals with OS who showed no brain malformations, indicating that mutated genes are involved in OS (Kato et al., 2007, 2009; Fullston et al., 2010; Giordano et al., 2010; Saito et al., 2008, 2010).

CASK (Genbank accession number NM_003688.3) at Xp11.4 encodes a calcium/calmodulin-dependent serine protein kinase of 921 amino acids belonging to the membrane-associated guanylate kinase protein family (Hsueh, 2006). Accumulating evidence indicates that *CASK* is essential for synapse formation at both presynaptic and postsynaptic junctions. In addition, *CASK* enters the nucleus and regulates expression of genes involved in cortical development (Hsueh, 2006). Recently, heterozygous loss-of-function mutations in *CASK* were found in four female patients with X-linked intellectual disability (ID);

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microcephaly and pontocerebellar hypoplasia (MICPCH) and a hemizygous synonymous c.915G>A mutation, which caused skipping of exon 9 of *CASK* in about 20% of the mutant transcripts, was found in a male patient with the same disease and presentation (Najm et al., 2008). To date, 32 additional female cases have been reported, suggesting that ID, MICPCH, growth retardation, axial hypotonia with or without hypertonia of extremities, and optic nerve hypoplasia are caused by loss-of-function mutations of *CASK* in female cases (Moog et al., 2011; Hayashi et al., 2012). On the other hand, a missense mutation causing a partial skipping of exon 2 of *CASK* was found in affected male individuals in an Italian family with FG syndrome, which is characterized by multiple congenital anomalies and ID (Piluso et al., 2009). More recently, five missense mutations and a splice mutation, causing amino acid changes or in-frame deletions of the *CASK* protein, were found in male patients and variably affected carrier female patients with ID, often accompanied by congenital nystagmus (Tarpey et al., 2009; Hackett et al., 2010). Therefore it has been postulated that hypomorphic *CASK* alleles cause ID in male individuals. Collectively, mutations of *CASK* could cause a wide spectrum of ID, ranging from nonsyndromic mild ID to syndromic severe ID with structural brain abnormalities in both male and female patients.

Herein, we report on two male patients with OS, cerebellar hypoplasia, and multiple congenital anomalies. One patient had a *CASK* deletion and the other had a mutation at the translation initiation codon, both likely leading to a loss of *CASK* function. Detailed clinical and molecular data are presented.

METHODS

Patients

A total of 34 Japanese patients (20 male and 14 female) with OS were analyzed for copy number aberrations. Twelve of them were additionally analyzed by whole exome sequencing. The diagnosis was made based on clinical features and characteristic patterns on EEG. Mutations in *STXBPI* were not identified in these patients (including Patients 1 and 2) by high-resolution melting analysis. Thirteen male patients, including Patient 1, and three female Patients were negative for *ARX* mutation. The experimental protocols were approved by the Yokohama City University School of Medicine Institutional Review Boards for Ethical Issues. Written informed consent was obtained from all individuals and/or their families in compliance with the relevant Japanese regulations.

Genomic microarray and cloning of deletion breakpoint

Genomic DNA obtained from peripheral blood leukocytes was used. Copy number alterations were studied by using Cytogenetics Whole-Genome 2.7M Array (Affymetrix, Santa Clara, CA, U.S.A.) for 30 patients and GeneChip

Human Mapping 250K NspI (Affymetrix) for four patients. Copy number alterations were analyzed using the Chromosome Analysis Suite (ChAS; Affymetrix) with NA30.1 (hg18) annotations (for 2.7M Array) or using CNAG2.0 (for 250K) (Nannya et al., 2005). The junction fragment spanning the deletion was amplified by long polymerase chain reaction (PCR), using several primer sets based on putative breakpoints from the microarray data. The junction fragment was amplified using following primers: forward, 5'-ACCCAGCGTTTCACCAAGGTCTCT-3'; reverse, 5'-GTGGCTTCAGAATTAGGCCACAAA-3' (product size = 1,136 bp). PCR products were electrophoresed in agarose gels, stained with ethidium bromide, extracted from the gels using a QIAquick Gel extraction kit (Qiagen, Tokyo, Japan), and sequenced.

Quantitative real-time PCR

The deletion of *CASK* was analyzed using the patient's and parental genomic DNA by quantitative real-time PCR (qPCR) on a Rotor-Gene Q thermal cycling system (Qiagen). DNA extracted from two independent blood samples each from the patient and mother were used for analysis. PCR was performed in a volume of 15 μ l containing 10 ng of genomic DNA, 1 \times Rotor-Gene SYBR Green PCR Master Mix (Qiagen), and 1.0 μ M each primer. qPCR was carried out using the two standard curve relative quantification method with four standard samples including 30, 10, 3.33, and 1.11 ng DNA, respectively. Three primer sets for exons 2, 3, and 4 of *CASK*, and one reference primer set for an area on chromosome 9 were used. Relative copy number of test regions was calculated in comparison with that of the reference region. The experiments were independently repeated three times. The data were averaged, and the standard deviation was calculated. Primer information is available on request.

Fluorescent in situ hybridization (FISH)

RP11-977L20 covering the deletion of *CASK* was labeled with SpectrumGreen -11-dUTP (Abbott, Tokyo, Japan) by nick translation. Probe-hybridization mixtures (15 μ l) were denatured at 70°C for 5 min, applied to chromosomes, incubated at 37°C for 20 h, and then washed and mounted with antifade solution (Vector Laboratories, Burlingame, CA, U.S.A.) containing 4,6-diamidino-2-phenylindole. Photographs were taken on an AxioCam MR Charge Coupled Device camera fitted to an Axioplan2 fluorescence microscope (Carl Zeiss, Tokyo, Japan). The mosaic ratio was examined by two independent investigators, who each counted 100 interphase nuclei.

RNA analysis

RNA analysis using lymphoblastoid cell lines was performed as described previously (Saitsu et al., 2011). Briefly, total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen); 2 μ g of total RNA was subjected to reverse transcription, and 1 μ l of cDNA was used for PCR.

Primer sequences are ex1-F (5'-ATGTGTACGAGCTGTGCGAGGTGAT-3') and ex4-R (5'-AGCGTCAGCTCGCTTTACGATTTCA-3'). Two separately extracted RNA samples were used in each duplicated experiment. The DNA in each PCR band was purified using a QIAquick Gel extraction kit (Qiagen) and sequenced.

Whole exome sequencing

DNAs were captured using the SureSelect^{XT} Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and sequenced with one lane per sample on an Illumina GAIIX platform (Illumina, San Diego, CA, U.S.A.) with 108-bp paired-end reads. Image analysis and base calling were performed by sequence control software real-time analysis and CASAVA software v1.7 (Illumina). A total of 94,106,348 paired-end reads were obtained for Patient 2 and aligned to the human reference genome sequence (GRCh37/hg19) using MAQ (Li et al., 2008) and NextGENe software v2.00 with sequence condensation by consolidation (SoftGenetics, State College, PA, U.S.A.). Single nucleotide variants (SNVs) were called using MAQ and NextGENe. Small insertions and deletions were detected using NextGENe. Called SNVs were annotated with SeattleSeq Annotation. The number of variants identified by exome sequencing in Patient 2 is shown in Table S1.

Immunoblotting

Lymphoblastoid cells were washed twice in ice-cold phosphate-buffered saline (PBS), and lysed in sodium dodecyl sulfate sample buffer. Samples were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to the polyvinylidene fluoride membrane, and analyzed with anti-CASK monoclonal antibody, which is produced by a synthetic peptide corresponding to residues surrounding Glu327 of human CASK protein (1:1,000 dilution, D24B12; Cell Signaling, Tokyo, Japan). Anti-Lamin B polyclonal antibody (1:500 dilution, sc-6217; Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) was used as a control. Secondary antibody was peroxidase-conjugated goat anti-rabbit IgG or bovine anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Blots were detected using the Supersignal West dura (Pierce, Yokohama, Japan). Chemiluminescence was visualized using a FluorChem 8900 (Alpha Innotech, San Leandro, CA, U.S.A.). Experiments were repeated twice using two separately prepared samples.

RESULTS

Clinical information

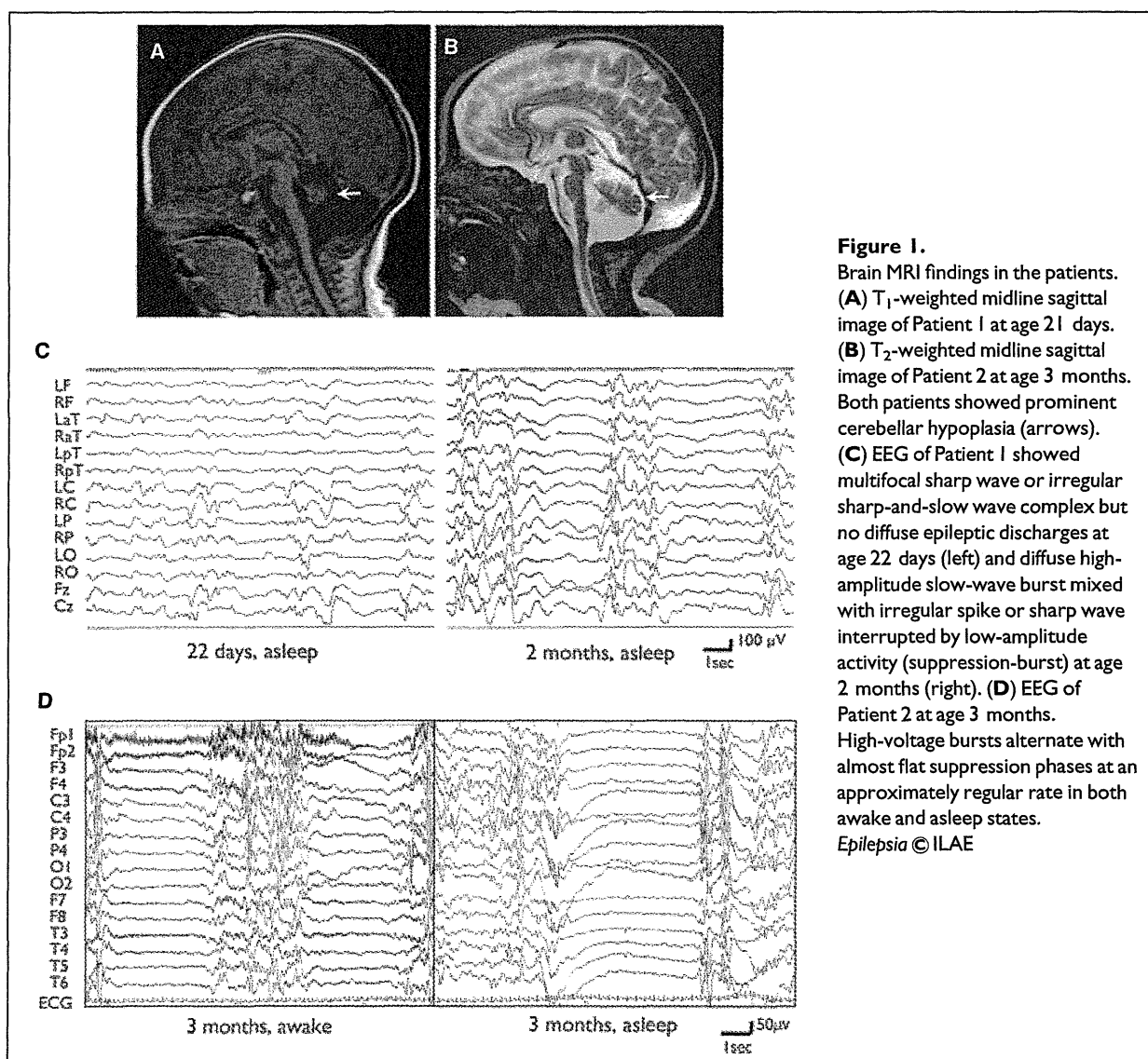
Patient 1 is a 4-year-old boy born to nonconsanguineous parents. The pregnancy was uneventful, and he was born at term (gestational age 41 weeks and 2 days) with induced labor but no asphyxia. His body weight was 2,606 g (−2.0 standard deviation [SD]), his height was 47.5 cm (−1.4 SD),

and his head circumference was 32.2 cm (−1.2 SD). An apneic event with cyanosis, which was not improved by positioning or oxygen inhalation, was evident 2 days after birth. Brain magnetic resonance imaging (MRI) demonstrated prominent cerebellar hypoplasia (Fig. 1A). EEG showed multifocal epileptic discharges with a short period (1 s) of flat basic rhythm (Fig. 1C, left). Phenobarbital was administered at 21 days and was effective for the apneic event. At the age of 2 months, he developed daily clustering of tonic seizures with suppression-burst pattern on both awake and asleep EEG (Fig. 1C, right) and poor feeding. EEG at 5 months demonstrated hypsarrhythmia, which is characteristically seen in West syndrome. He exhibited long slender fingers, micropenis, micrognathia, and a short neck with obstructive respiration, and then required tracheostomy with laryngotracheal separation and gastrostomy. His head circumference was 47.1 cm (−2.7 SD) at 1 years and 4 months. On examination at 4 years, he was bedridden and unable to track objects. Tonic seizures lasting 10–30 s several times a day and frequent myoclonic seizures were seen regardless of treatment with phenobarbital, pyridoxal phosphate, zonisamide, clobazam, and lamotrigine. EEG during sleep at 3 years of age demonstrated multifocal sharp and slow-wave complexes and diffuse low-voltage fast-wave bursts or a desynchronization pattern.

Patient 2 is a 4-year-old boy born to nonconsanguineous parents. He was born at 39 weeks of gestation without asphyxia after uneventful pregnancy. His body weight was 2,000 g (−3.3 SD), his height was 43.0 cm (−2.8 SD), and his head circumference was 29.5 cm (−2.7 SD). He was poorly fed with milk and referred to us at 27 days after birth. Multiple anomalies were recognized such as micrognathia, high arched palate, shortened upper arms, bilateral overlapping fingers and clinodactyly, and persistent hypertrophic primary vitreous. He underwent ophthalmic surgery at 33 days after birth. Brain MRI demonstrated prominent cerebellar hypoplasia (Fig. 1B). At 3 months of age, he showed frequent generalized tonic seizures, and EEG showed a suppression-burst pattern in both awake and asleep states (Fig. 1D). He showed normal auditory brain responses. Laboratory data, including lactate, pyruvate, and very long fatty acids, were all normal. Phenobarbital was initiated and only partially effective for his seizures. Topiramate, clobazam, and sodium bromide were added, and seizure frequencies were decreased from daily to weekly. His development was severely delayed with no head control or eye pursuit. His deep tendon reflexes are exaggerated, with positive bilateral Babinski signs. He shows muscle hypertonus with rigidity of both upper and lower limbs.

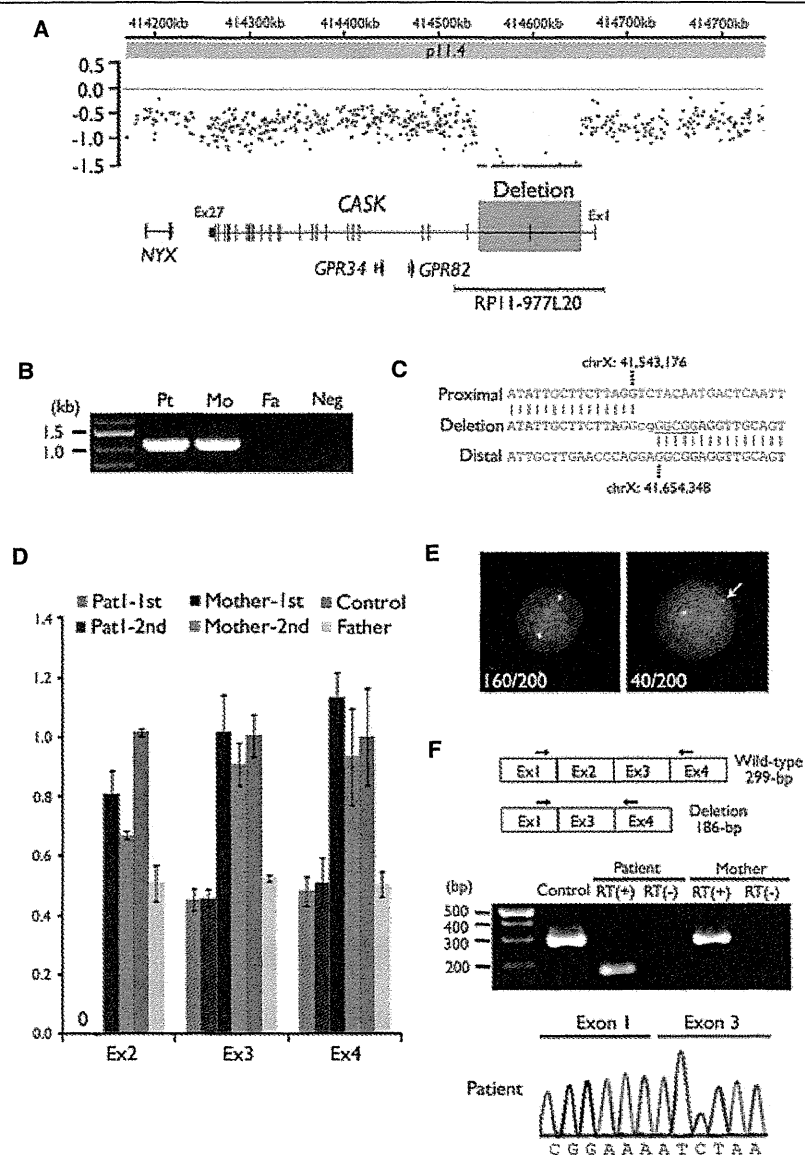
Copy number analysis

Through screening for copy number alterations by genomic microarray analysis, we identified an approximately 110-kb microdeletion involving exon 2 of CASK at Xp11.4 in Patient 1 (Fig. 2A). Breakpoint-specific PCR analysis of



the family showed that the deletion was inherited from his mother (Fig. 2B). The sequence of the junctional fragment confirmed a 111,172-bp deletion (NG_016754.1: g.17883_129055del) (Fig. 2C). Sequencing also identified 5-bp duplicated sequences as well as a 2-bp insertion at the deletion junction. We were surprised that the healthy mother possessed this deletion, because the deletion is predicted to lead to a frameshift with presumably premature termination of the translation. The deletion was further examined by qPCR and FISH analyses. Whereas the relative copy numbers of exons 3 and 4 (not deleted) were nearly 1.0 in the two maternal DNA samples, as expected, those for deleted exon 2 in the two samples were 0.67 and 0.81 (Fig. 2D). Because the relative copy number is expected to be 0.5 if one of two copies is deleted (as the healthy father showed), this result suggested that the mother may be

somatic mosaic for the deletion. In fact, FISH analysis revealed that only 40 of 200 interphase nuclei showed one clear signal and another weaker signal, consistent with partial deletion within the bacterial artificial chromosome probe (Fig. 2E). Based on these findings, we concluded that the mother is somatic mosaic for the deletion, and that the percentage of mosaicism is approximately 20%. To explore the effect of the deletion on the transcription of *CASK*, reverse transcriptase PCR designed to amplify exons 1–4 was performed using total RNA extracted from lymphoblastoid cell lines (LCLs) derived from the patient and his mother (Fig. 2F). A single band (299-bp) corresponding to the wild-type *CASK* allele was amplified using a complementary DNA (cDNA) template from a control LCL (Fig. 2F). By contrast, only a smaller band, in which exon 2 had been deleted, was detected from the patient's cDNA

**Figure 2.**

A 111-kb deletion involving exon 2 of *CASK*. (A) The 2.7M array profile clearly shows a deletion involving exon 2 of *CASK* at Xp11.4. The x- and y-axes show the genomic location from the p telomere of chromosome X (UCSC coordinates, May, 2006) and \log_2 signal ratio values, respectively. Four RefSeq genes including *CASK* and RPI1-977L20 clone used for FISH are shown. (B) Breakpoint-specific PCR analysis of the family. Primers flanking the deletion were able to amplify a 1,136-bp product from both the Patient 1 and his mother. Pt, patient; Mo, mother; Fa, Father; Neg, negative control (no template DNA). (C) Deletion junction sequence. Top, middle, and bottom strands show proximal, deleted and distal sequences, respectively. The two nucleotides inserted are presented in lower case. A 5-bp sequence that appears twice at the breakpoint region is colored red or underlined. (D) qPCR analysis of the family, and a female control. Two DNA samples extracted from two independent blood samples were used for analysis of the patient and his mother. Relative copy numbers of deleted exon 2 were 0.67 and 0.81 (both above 0.5) in the mother, suggesting somatic mosaicism of the deletion. (E) FISH images of RPI1-977L20, covering the deletion, on the mother's chromosomes. One-hundred sixty nuclei showed two clear signals (left), and 40 nuclei showed one clear signal and a weaker signal (right, white arrow) consistent with partial deletion within the probe. (F) Schematic representation of the transcript from exons 1–4 of *CASK*. Exons and primers are depicted as boxes and arrows, respectively (top). A single wild-type amplicon was detected in a control and the mother. A smaller product was amplified only from the patient's cDNA. RT (+): with reverse transcriptase, RT (-): without reverse transcriptase as a negative control. Sequence of a smaller amplicon clearly demonstrated the exon 2 deletion (bottom).

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(Fig. 2F). The smaller mutant band was not detected from the mother's cDNA (Fig. 2F). Human androgen receptor assay showed that X-inactivation was random (70:30) in the mother (data not shown). However, because the percentage of mosaicism was low (20%), it remains possible that the deletion allele may undergo X-inactivation in cells possessing it, leading to diminished expression of the deletion allele in LCL.

Whole exome sequencing

To find potential pathologic mutations, whole exome sequencing of 12 patients was performed. We focused on mutations in *CASK*, and identified a hemizygous c.1A>G mutation of the first ATG codon in Patient 2 (Fig. 3A,B). This mutation is anticipated to result in alternative ATG codon usage. By using the next downstream in-frame ATG codon positioned at c.202_204 (Fig. 3C), a truncated protein without the first 67 amino acids containing calmodulin-dependent kinase domain could be produced, although this ATG codon (CATATGC) does not conform to the Kozak

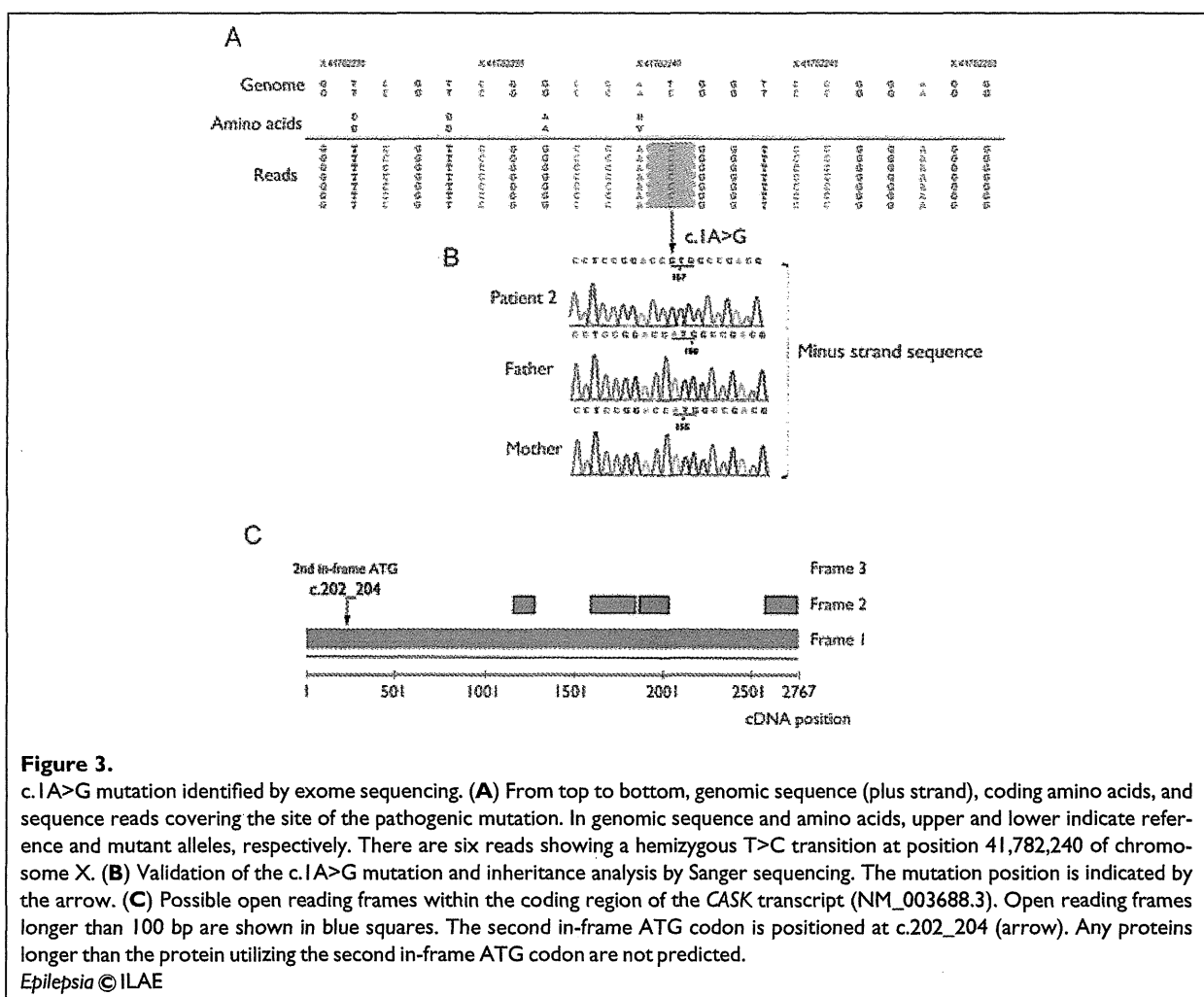
consensus. The parental DNA did not have the mutation, suggesting that the mutation occurred de novo (Fig. 3B). No *CASK* mutations were found in any of the other patients.

Immunoblotting

To evaluate mutational effect for *CASK* expression in two patients, immunoblotting was performed using total lysate of LCL. A strong signal at 104 kDa was detected in a control and the mother of Patient 1, showing strong expression of wild-type *CASK* protein in LCLs (Fig. 4, top). However, both Patients 1 and 2 did not show any detectable signal (Fig. 4, top), whereas the Lamin B showed comparable expression in all samples loaded (Fig. 4, bottom). Thus these data suggest that expression of *CASK* protein was severely decreased in two patients.

DISCUSSION

We describe two male patients possessing an intragenic *CASK* deletion (only exon 2) or a hemizygous c.1A>G



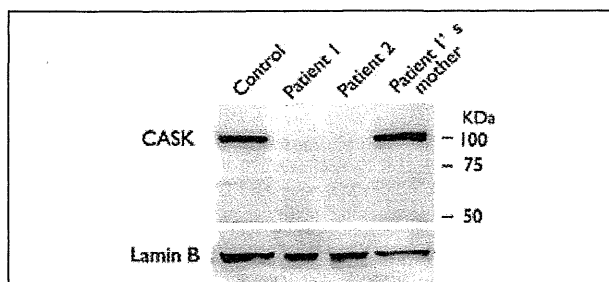


Figure 4.

Expression of *CASK* protein in LCL. Immunoblot analysis by using a monoclonal *CASK* antibody (top). Expression of *CASK* protein was not detected in LCL derived from two patients, whereas LCL of a control and Patient 1's mother showed strong *CASK* expression. The observed differences in expression were not due to difference of loading conditions, because the level of Lamin B protein was similar in all cases (bottom).

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mutation. In Patient 1, the deletion is likely to be an almost null mutation as the mutant *CASK* transcript with exon 2 deletion has a frameshift with premature termination. Deletions in *CASK* have been reported in 16 female patients, and a skewed X-inactivation pattern was observed in two of them (the others had random inactivation pattern or not determined) (Froyen et al., 2007; Hayashi et al., 2008; Najm et al., 2008; Moog et al., 2011; Hayashi et al., 2012). Of interest, partial skipping of the exon 2 of *CASK* (approximately 3–6% of the unskipped transcripts) has been reported in male patients with FG syndrome showing ID, relative macrocephaly, hypotonia, severe constipation, and behavioral disturbance (Piluso et al., 2003, 2009). By contrast, our Patient 1 with complete deletion of exon 2 showed a more severe phenotype, suggesting that he showed one of the most severe phenotypes caused by *CASK* abnormalities. In Patient 2, the mutation of the first ATG codon could produce a truncated protein without the amino terminal 67 amino acids. However, this alternative in-frame ATG codon does not conform to the Kozak consensus, suggesting that its translation would be significantly reduced. In fact, *CASK* protein was not detected in the LCL of two patients, suggesting that expression of *CASK* protein should be extremely low. Because only partial skipping of exon 9 (about 20% of the mutant transcripts) (Najm et al., 2008) or of exon 2 (3–6% of the unskipped transcripts) (Piluso et al., 2009) is sufficient to cause ID and other features in male cases, it is likely that the maintenance of expression level of functional *CASK* protein is essential.

Two male patients with *CASK* abnormalities showed typical OS features, revealing an association between OS and *CASK* abnormalities in male patients, which has to date never been shown. Microcephaly and prominent cerebellar hypoplasia were also recognized, consistent with previous

reports (Najm et al., 2008; Moog et al., 2011; Hayashi et al., 2012). Of interest, our patients also showed reduced body size and multiple congenital anomalies such as high arched palate, micrognathia, finger anomalies, and persistent hypertrophic primary vitreous. This suggests that *CASK* may be involved in overall body growth and development of these organs in humans. Supporting this idea, growth retardation and small jaw have been reported in patients with *CASK* abnormalities (Najm et al., 2008; Hackett et al., 2010; Moog et al., 2011). In addition, *CASK*-deficient mice showed micrognathia and cleft palate with male lethality (Laverty & Wilson, 1998), and hypomorphic *CASK* mutant mice are significantly smaller than littermate control mice (Atasoy et al., 2007). Therefore, it is likely that loss-of-function mutations in *CASK* cause reduced body size and multiple congenital anomalies, as well as OS and cerebellar hypoplasia.

The same deletion was found in both the mother and the affected son, indicating a germline mosaicism in the mother associated with recurrence risks. This information is useful for genetic counseling in the family. The maternal somatic mosaicism was confirmed by different methods including FISH, qPCR, and breakpoint-specific PCR analyses. We would like to emphasize the importance of breakpoint-specific PCR analysis, in which a specific band undoubtedly indicates the presence of the deletion allele. Because PCR is a powerful tool for amplifying target sequences, we could easily detect the somatic mosaic, even though it existed in approximately 20% of cells. In addition, it has been reported that PCR analyses of the deletion junction can detect extremely low-level mosaicism not detected by array comparative genomic hybridization (Zhang et al., 2009). The increasing density of available oligonucleotide arrays allows us to design long (or even regular) PCR primers for junctional cloning. Once junctional cloning is successful (though it is sometimes difficult), it is highly useful for examining parental states.

It has been determined that mutations in three genes (*STXBPI*, *ARX*, and *CASK*) cause OS. Screening for *STXBPI* mutations should be considered in OS patients with no brain anomalies in both male and female patients. Screening for *ARX* mutations would be reasonable in male patients with OS, and the presence of micropenis may encourage its screening (Kato et al., 2007). Based on this study, *CASK* mutations should be considered in patients with OS and cerebellar hypoplasia.

In conclusion, we report for the first time *CASK* abnormalities in male individuals with OS. Maternal somatic mosaicism of a *CASK* deletion is also described, suggesting that somatic and germline mosaicism of a microdeletion should be carefully considered in the examination of parental samples. Our data expand the clinical spectrum of *CASK* mutations to include OS with cerebellar hypoplasia and congenital anomalies at the most severe end of clinical presentation.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

REFERENCES

- Atasoy D, Schoch S, Ho A, Nadasy KA, Liu X, Zhang W, Mukherjee K, Nosyreva ED, Fernandez-Chacon R, Missler M, Kavalali ET, Sudhof TC. (2007) Deletion of CASK in mice is lethal and impairs synaptic function. *Proc Natl Acad Sci U S A* 104:2525–2530.
- Djukic A, Lado FA, Shinnar S, Moshe SL. (2006) Are early myoclonic encephalopathy (EME) and the Ohtahara syndrome (EIEE) independent of each other? *Epilepsy Res* 70(Suppl. 1):S68–S76.
- Froyen G, Van Esch H, Bauters M, Hollanders K, Frints SG, Vermeesch JR, Devriendt K, Fryns JP, Marynen P. (2007) Detection of genomic copy number changes in patients with idiopathic mental retardation by high-resolution X-array-CGH: important role for increased gene dosage of XLMR genes. *Hum Mutat* 28:1034–1042.
- Fullston T, Brueton L, Willis T, Philip S, MacPherson L, Finnis M, Gez C, Morton J. (2010) Ohtahara syndrome in a family with an ARX protein truncation mutation (c.81C>G/p.Y27X). *Eur J Hum Genet* 18:157–162.
- Giordano L, Sartori S, Russo S, Accorsi P, Galli J, Tiberti A, Bettella E, Marchi M, Vignoli A, Darra F, Murgia A, Bernardina BD. (2010) Familial Ohtahara syndrome due to a novel ARX gene mutation. *Am J Med Genet A* 152A:3133–3137.
- Hackett A, Tarpey PS, Licata A, Cox J, Whibley A, Boyle J, Rogers C, Grigg J, Partington M, Stevenson RE, Tolmie J, Yates JR, Turner G, Wilson M, Futreal AP, Corbett M, Shaw M, Gez C, Raymond FL, Stratton MR, Schwartz CE, Abidi FE. (2010) CASK mutations are frequent in males and cause X-linked nystagmus and variable XLMR phenotypes. *Eur J Hum Genet* 18:544–552.
- Hayashi S, Mizuno S, Migita O, Okuyama T, Makita Y, Hata A, Imoto I, Inazawa J. (2008) The CASK gene harbored in a deletion detected by array-CGH as a potential candidate for a gene causative of X-linked dominant mental retardation. *Am J Med Genet A* 146A:2145–2151.
- Hayashi S, Okamoto N, Chinen Y, Takanashi JI, Makita Y, Hata A, Imoto I, Inazawa J. (2012) Novel intragenic duplications and mutations of CASK in patients with mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH). *Hum Genet* 131:99–110.
- Hsueh YP. (2006) The role of the MAGUK protein CASK in neural development and synaptic function. *Curr Med Chem* 13:1915–1927.
- Kato M, Saitoh S, Kamei A, Shiraishi H, Ueda Y, Akasaka M, Tohyama J, Akasaka N, Hayasaka K. (2007) A longer polyalanine expansion mutation in the ARX gene causes early infantile epileptic encephalopathy with suppression-burst pattern (Ohtahara Syndrome). *Am J Hum Genet* 81:361–366.
- Kato M, Koyama N, Ohta M, Miura K, Hayasaka K. (2009) Frameshift mutations of the ARX gene in familial Ohtahara syndrome. *Epilepsia* 51:1679–1684.
- Lavery HG, Wilson JB. (1998) Murine CASK is disrupted in a sex-linked cleft palate mouse mutant. *Genomics* 53:29–41.
- Li H, Ruan J, Durbin R. (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Res* 18:1851–1858.
- Moog U, Kutsche K, Kortum F, Chilian B, Bierhals T, Apeshiotis N, Balg S, Chassaing N, Coubes C, Das S, Engels H, Van Esch H, Grasshoff U, Heise M, Isidor B, Jarvis J, Koehler U, Martin T, Oehl-Jaschkowitz B, Ortibus E, Pilz DT, Prabhakar P, Rappold G, Rau I, Rettenberger G, Schluter G, Scott RH, Shoukier M, Wohlleber E, Zirm B, Dobyns WB, Uyanik G. (2011) Phenotypic spectrum associated with CASK loss-of-function mutations. *J Med Genet* 48:741–751.
- Najm J, Horn D, Wimplinger I, Golden JA, Chizhikov VV, Sudi J, Christian SL, Ullmann R, Kuechler A, Haas CA, Flubacher A, Charnas LR, Uyanik G, Frank U, Klopocki E, Dobyns WB, Kutsche K. (2008) Mutations of CASK cause an X-linked brain malformation phenotype with microcephaly and hypoplasia of the brainstem and cerebellum. *Nat Genet* 40:1065–1067.
- Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S. (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res* 65:6071–6079.
- Ohtahara S, Yamatogi Y. (2006) Ohtahara syndrome: with special reference to its developmental aspects for differentiating from early myoclonic encephalopathy. *Epilepsy Res* 70(Suppl. 1):S58–S67.
- Ohtahara S, Ishida T, Oka E, Yamatogi Y, Inoue H, Karita S, Ohtsuka Y. (1976) [On the specific age dependent epileptic syndrome: the early-infantile epileptic encephalopathy with suppression-burst.]. *No to Hattatsu* 8:270–279.
- Piluso G, Carella M, D'Avanzo M, Santinelli R, Carrano EM, D'Avanzo A, D'Adamo AP, Gasparini P, Nigro V. (2003) Genetic heterogeneity of FG syndrome: a fourth locus (FGS4) maps to Xp11.4-p11.3 in an Italian family. *Hum Genet* 112:124–130.
- Piluso G, D'Amico F, Saccone V, Bismuto E, Rotundo IL, Di Domenico M, Aurino S, Schwartz CE, Neri G, Nigro V. (2009) A missense mutation in CASK causes FG syndrome in an Italian family. *Am J Hum Genet* 84:162–177.
- Saitsu H, Kato M, Mizuguchi T, Hamada K, Osaka H, Tohyama J, Urano K, Kumada S, Nishiyama K, Nishimura A, Okada I, Yoshimura Y, Hirai S, Kumada T, Hayasaka K, Fukuda A, Ogata K, Matsumoto N. (2008) De novo mutations in the gene encoding STXBPI (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat Genet* 40:782–788.
- Saitsu H, Kato M, Okada I, Orii KE, Higuchi T, Hoshino H, Kubota M, Arai H, Tagawa T, Kimura S, Sudo A, Miyama S, Takami Y, Watanabe T, Nishimura A, Nishiyama K, Miyake N, Wada T, Osaka H, Kondo N, Hayasaka K, Matsumoto N. (2010) STXBPI mutations in early infantile epileptic encephalopathy with suppression-burst pattern. *Epilepsia* 51:2397–2405.
- Saitsu H, Hoshino H, Kato M, Nishiyama K, Okada I, Yoneda Y, Tsurusaki Y, Doi H, Miyake N, Kubota M, Hayasaka K, Matsumoto N. (2011) Paternal mosaicism of an STXBPI mutation in OS. *Clin Genet* 80:484–488.
- Tarpey PS, Smith R, Pleasance E, Whibley A, Edkins S, Hardy C, O'Meara S, Latimer C, Dicks E, Menzies A, Stephens P, Blow M, Greenman C, Xue Y, Tyler-Smith C, Thompson D, Gray K, Andrews J, Barthorpe S, Buck G, Cole J, Dunmore R, Jones D, Maddison M, Mironenko T, Turner R, Turrell K, Varian J, West S, Widaa S, Wray P, Teague J, Butler A, Jenkinson A, Jia M, Richardson D, Shepherd R, Wooster R, Tejada MI, Martinez F, Carvill G, Goliath R, de Brouwer APM, van Bokhoven H, Van Esch H, Chelly J, Raynaud M, Ropers H-H, Abidi FE, Srivastava AK, Cox J, Luo Y, Mallya U, Moon J, Parnau J, Mohammed S, Tolmie JL, Shoubridge C, Corbett M, Gardner A, Haan E, Rujirabanjerd S, Shaw M, Vandeleur L, Fullston T, Easton DF, Boyle J, Partington M, Hackett A, Field M, Skinner C, Stevenson RE,

- Bobrow M, Turner G, Schwartz CE, Geetz J, Raymond FL, Futreal PA, Stratton MR. (2009) A systematic, large-scale resequencing screen of X-chromosome coding exons in mental retardation. *Nat Genet* 41:535–543.
- Yamatogi Y, Ohtahara S. (2002) Early-infantile epileptic encephalopathy with suppression-bursts, Ohtahara syndrome; its overview referring to our 16 cases. *Brain Dev* 24:13–23.
- Zhang F, Khajavi M, Connolly AM, Towne CF, Batish SD, Lupski JR. (2009) The DNA replication FoSTeS/MMBIR mechanism can generate genomic, genic and exonic complex rearrangements in humans. *Nat Genet* 41:849–853.

Table S1. All variants identified by exome sequencing in Patient 2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Reply

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We thank Drs Jellinger and Attems for their interest in our study. In agreement with prior reports, we found that Parkinson disease (PD) pathology, including nigral neuronal loss and Lewy body pathology, is common in older adults without PD. Furthermore, we provide evidence that PD nigral pathology is related to parkinsonian motor signs in persons without a clinical diagnosis of PD.¹ This contrasts with prior studies of incidental Lewy body disease, which found associations with subtle electrophysiologic changes but not with overt motor signs.² Interestingly, in the current study, we also found that Alzheimer disease (AD) and cerebrovascular pathology showed independent associations with the severity of parkinsonian motor signs.¹ As requested, the correlations among these common brain pathologies are included in the accompanying Table. It is interesting that Dr Attems and colleagues did not find an association of nigral pathology or cerebrovascular disease with parkinsonian signs among persons with AD.³ We and others have reported such associations.^{4–6} Overall, the findings in the current study have important public health implications. They suggest that mild parkinsonian signs, reported in up to 50% of older adults by age 85 years and associated with significant morbidity and mortality, may be caused by a range of pathologies including PD pathology, AD, and cerebrovascular pathologies. These data underscore the need for more sensitive clinical measures and biomarkers that can detect and differentiate the various neuropathologies underlying the development of parkinsonian signs in old age.

Potential Conflicts of Interest

Nothing to report.

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References

1. Buchman AS, Shulman JM, Nag S, et al. Nigral pathology and parkinsonian signs in elders without Parkinson disease. *Ann Neurol* 2012;71:258–266.
2. Caviness JN. Presymptomatic Parkinson's disease: the Arizona experience. *Parkinsonism Relat Disord* 2012;18(suppl 1):S203–S206.
3. Attems J, Quass M, Jellinger K. Tau and α -synuclein brainstem pathology in Alzheimer disease: relation with extrapyramidal signs. *Acta Neuropathol* 2007;113:53–62.
4. Burns JM, Galvin JE, Roe CM, et al. The pathology of the substantia nigra in Alzheimer disease with extrapyramidal signs. *Neurology* 2005;64:1397–1403.
5. Schneider JA, Li JL, Li Y, et al. Substantia nigra tangles are related to gait impairment in older persons. *Ann Neurol* 2006;59:166–173.
6. Buchman AS, Leurgans SE, Nag S, et al. Cerebrovascular disease pathology and parkinsonian signs in old age. *Stroke* 2011;42:3183–3189.

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Whole Exome Sequencing Identifies *KCNQ2* Mutations in Ohtahara Syndrome

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Recently, Weckhuysen et al revealed that *KCNQ2* mutations are involved in a substantial proportion of patients with a neonatal epileptic encephalopathy.¹ Some cases showed a suppression–burst pattern on electroencephalogram (EEG), tonic seizures, and profound intellectual disability, resembling Ohtahara syndrome (OS). By whole exome sequencing analysis of 12

TABLE: Intercorrelation of Postmortem Indices

Index	Macroinfarcts	Microinfarcts	Arteriolosclerosis	AD Pathology	Nigral Lewy Bodies
Nigral neuronal loss	0.07, 0.068	0.02, 0.628	0.13, <0.001	0.14, <0.001	0.38, <0.001
Macroinfarcts	—	0.39, 0.056	0.26, <0.001	0.09, 0.017	–0.063, 0.072
Microinfarcts		—	0.15, <0.001	0.04, 0.315	–0.10, 0.075
Arteriolosclerosis			—	0.03, 0.385	0.03, 0.491
AD pathology				—	0.07, 0.052

Based on Spearman or tetrachoric correlation and *p* value.

TABLE: Summary of the Clinical Features of Subjects with KCNQ2 Mutations

Case #	Mutation	Sex	Age	Age at Onset, Days	Initial Symptoms	Initial Epileptic Attacks	Initial EEG	Age at Onset of Spasms, Days	Age at Onset of SB Pattern, Days	Response to Therapy	Other Drugs Used, but Ineffective	Development	Neurological Examination	Involuntary Movement
1469	c.1010C>G (p.A337G) de novo	M	7 years	7	Vomiting	7 days, tonic seizure	SB	—	22	Seizure free and SB on EEG, disappeared after high-dose PB, CPS since age 5 years	B6, ZNS	No meaningful words, able to crawl, stand with support	Severe MR, no pyramidal signs	No
1654	c.341C>T (p.T114I) de novo	F	7 years	0	Tremor of the upper extremities	2 days, generalized convulsion with cyanosis	SB	—	2	Seizure free after ZNS, CPS since age 5 years	B6, CZP; PHT	DQ 10, bed-ridden, smiling	Profound MR, spastic quadriplegia	No
1754	c.794C>T (p.A265V) de novo	M	3 months	1	Apneic spell	1 days, tonic spasms with right opsoclonuslike movement	SB	1	2	Intractable	B6, ZNS; VPA, CZP, CBZ	Delayed, no eye pursuit	Unknown	Myoclonus at the bilateral upper extremities

B6 = vitamin B6; CBZ = carbamazepine; CPS = complex partial seizures; CZP = clonazepam; DQ = developmental quotient; EEG = electroencephalogram; MR = mental retardation; PB = phenobarbital; PHT = phenytoin; SB = suppression-burst; VPA = valproic acid; ZNS = zonisamide.

patients with OS, we found 3 missense mutations in *KCNQ2* (25%): c.341C>T (p.T114I), c.1010C>G (p.A337G), and c.794C>T (p.A265V) in 3 patients. All 3 patients showed initial seizures early in the neonatal period and a characteristic suppression-burst pattern on EEG, leading to diagnosis as OS (Table). Seizures were temporarily well controlled in 2 patients. Consistent with Weckhuysen's report, in which 6 of 8 mutations arose de novo, the 3 mutations in our series are de novo changes. Thus, it is likely that de novo *KCNQ2* mutations are among the common causes of early onset epileptic encephalopathies, including OS. *KCNQ2* mutations have been shown to cause benign familial neonatal seizures, which is distinct from OS.^{2,3} We unexpectedly found *KCNQ2* mutations by whole exome sequencing. Exome sequencing using familial trios (patients and their parents) can identify de novo mutations.⁴ Novel associations between unexpected gene mutations and early onset epileptic encephalopathies may be validated by such new technologies.

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Potential Conflicts of Interest

Nothing to report.

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References

- Weckhuysen S, Mandelstam S, Suls A, et al. KCNQ2 encephalopathy: emerging phenotype of a neonatal epileptic encephalopathy. *Ann Neurol* 2012;71:15–25.
- Singh NA, Charlier C, Stauffer D, et al. A novel potassium channel gene, *KCNQ2*, is mutated in an inherited epilepsy of newborns. *Nat Genet* 1998;18:25–29.

3. Biervert C, Schroeder BC, Kubisch C, et al. A potassium channel mutation in neonatal human epilepsy. *Science* 1998;279:403–406.
4. Vissers LE, de Ligt J, Gilissen C, et al. A de novo paradigm for mental retardation. *Nat Genet* 2010;42:1109–1112.

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Brain Death in Children: Why Does It Have to Be So Complicated?

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The authors appreciate the editorial comments by Wijdicks and Smith¹ and would like to address concerns about why the diagnosis of brain death in pediatric patients has to be “so complicated.”

This revised clinical guideline focused specifically on determining brain death and deliberately excluded issues related to ethical concerns and organ donation. Failure to mention the Child Neurology Society (CNS) as the third sponsoring society of this guideline is a major oversight of the editorial.¹ CNS provided significant review by Practice Committee members and the society’s Executive Board.² The quality of evidence provided in this guideline was equivalent to, if not more comprehensive than, the revised American Academy of Neurology (AAN) guideline, which reported only class III or IV evidence for 4 of 5 questions posed.³ We used the GRADE system to develop a consensus guideline because no class I or II studies to determine pediatric brain death exist.² Interestingly, the AAN is currently revising guideline development for practicing neurologists to use a modification of the GRADE system.

A wide range of clinical entities can result in brain death in newborns, children, and adolescents. The guideline, the checklist, and Table 3 clearly state that all reversible conditions should be excluded prior to the first brain death examination. However, some uncertainty in the newborn period still exists leading to age-based observation periods. These consensus based recommendations reflect extensive clinical experience across several pediatric disciplines. Additionally, provisions for pediatric trauma patients and neonates were included. Virtually every committee member has cared for acutely injured children who met examination criteria for brain death within the initial 24 hours. Some recovered brain function although most did not which is why 2 examinations over defined time periods is recommended. The recommended time periods are consensus based rather than arbitrary time periods. Neurologic examination findings remaining unchanged and consistent with brain death throughout the observation period was one of the recommended criteria for determining brain death in the 1987 guidelines. The committee retained this recommendation in the current update. We agree that apparent neurologic improvements reported in anecdotal cases are due to diagnostic errors when critically examined; this is precisely the reason why a change in findings between examinations implies the neurological process is potentially reversible, precluding the diagnosis of brain death.

The revised guideline repeatedly states that brain death is a clinical diagnosis, and factors influencing the neurologic

examination must be corrected before initiating brain death evaluation and apnea testing. Ancillary studies do not trump the neurological examination, and we clearly state that ancillary studies should not be viewed as a substitute for the neurologic examination. However, situations exist where ancillary studies are helpful to determine death. The revised guideline and checklist have simplified and clarified many previous sources of confusion. Additionally, the checklist will help standardize determination and documentation of brain death in children.⁴

Prolonging declaration of death does not appear to be a major concern in children—perhaps differing from the experience in adults. Families appreciate the added certainty conferred by the second examination. Patients in children’s hospitals rely on assessments by pediatric specialists who understand the unique needs of children and their families. The approach to caring for children is very different and likely more family centered. These issues are further addressed in the full guideline and we encourage readers to review the entire document published in *Critical Care Medicine and Pediatrics*.^{2,5}

Declaring brain death in children is complicated and should be undertaken by physicians who are adequately trained in the complexities involved in this important determination. We agree more research is needed to address some of the other issues raised in the editorial, and we again thank Drs Wijdicks and Smith for their opinion.

Potential Conflicts of Interest

Nothing to report.

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References

1. Wijdicks EF, Smith WS. Brain death in children: why does it have to be so complicated? *Ann Neurol* 2012;71:442–443.
2. Nakagawa TA, Ashwal S, Mathur M, et al. Guidelines for the determination of brain death in infants and children: an update of the 1987 Task Force recommendations. *Crit Care Med* 2011;39:2139–2155.
3. Wijdicks EF, Varelas PN, Gronseth GS, et al. Evidence-based guideline update: determining brain death in adults: report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2010;74:1911–1918.
4. Fackler J, Goldstein B. Pediatric brain death. *Crit Care Med* 2011;39:2197–2198.
5. Clinical report - Guidelines for the Determination of Brain Death in Infants and Children. An Update of the 1987 Task Force Recommendations. Nakagawa TA, Ashwal SA, Mathur M, Mysore M., and the Committee for Brain Death in Infants and Children. *Pediatrics*. 2011;128:3 e720-e740. doi: 10.1542/peds.2011-1511.

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KDM6A Point Mutations Cause Kabuki Syndrome

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ABSTRACT: Kabuki syndrome (KS) is a rare congenital anomaly syndrome characterized by a unique facial appearance, growth retardation, skeletal abnormalities, and intellectual disability. In 2010, *MLL2* was identified as a causative gene. On the basis of published reports, 55–80% of KS cases can be explained by *MLL2* abnormalities. Recently, de novo deletion of *KDM6A* has been reported in three KS patients, but point mutations of *KDM6A* have never been found. In this study, we investigated *KDM6A* in 32 KS patients without an *MLL2* mutation. We identified two nonsense mutations and one 3-bp deletion of *KDM6A* in three KS cases. This is the first report of *KDM6A* point mutations associated with KS.

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KEY WORDS: Kabuki syndrome; *KDM6A*; point mutations; chromosome X

Kabuki syndrome (KS; MIM# 147920), first described by Niikawa and Kuroki in 1981, is a rare congenital anomaly syndrome with the characteristic facial features of a long palpebral fissure and eversion of lateral third of the inferior eyelids [Kuroki et al., 1981; Niikawa et al., 1981]. Individuals with KS also show mild to severe intellectual disability, growth retardation, skeletal abnormalities, and a variety of visceral malformations. Although KS is thought to inherit in autosomal dominant fashion, other inheritance patterns have also been considered [Matsumoto and Niikawa, 2003]. In 2010, whole exome sequencing successfully identified loss-of-function mutations in *MLL2* in KS. *MLL2* maps to 12q13.12 and consists of at least 54 coding exons. *MLL2* encodes a histone H3 lysine 4 (H3K4)-specific

methyl transferase and plays important roles in the epigenetic control of active chromatin states. On the basis of recent reports of *MLL2* mutations in KS, the mutation detection rate of *MLL2* in KS is 55–80% [Banka et al., 2012]. Among the published mutations, 73.2% (170/232) were truncation type, and pathogenic missense mutations were mainly localized in exon 48 [Banka et al., 2012].

X-linked inheritance has also been implicated in KS. Sex chromosome abnormalities in KS have been reported many times and some of the clinical manifestations are shared with Turner syndrome; patients showing overlapping features, called “Turner–Kabuki” syndrome, have been reported [Bianca et al., 2009; Dennis et al., 1993; Niikawa et al., 1988; Rodriguez et al., 2008; Stankiewicz et al., 2001; Wellesley and Slaney, 1994]. Common structural abnormalities (inversion, translocation, and ring chromosome) involving Xp11 and Yp11 in the pseudoautosomal region were observed in KS, implying the potential involvement of the regions for pathogenesis in KS [Matsumoto and Niikawa, 2003]. In addition, two unrelated KS patients with ring X (p11.2q13) have been reported [McGinniss et al., 1997; Niikawa et al., 1988]. However, an X-linked gene for KS has not been identified until recently. In 2012, complete or partial de novo deletions of *KDM6A* (MIM# 300228) were identified in three patients with KS [Lederer et al., 2012]. *KDM6A* resides at Xp11.3 and encodes the lysine demethylase 6A (*KDM6A*) demethylating di- and trimethyl-lysine 27 on histone H3 (H3K27) [Lee et al., 2007]. H3K4 methylation by *MLL2/3* is linked to the demethylation of H3K27 by *KDM6A* [Lee et al., 2007]. These authors sequenced *KDM6A* in their series of 22 patients, but found no point mutations [Lederer et al., 2012]. In this study, we investigated *KDM6A* with regard to point mutations in KS after obtaining written informed consents from families of patients. The institutional review board of Yokohama City University School of Medicine approved this study.

To identify *KDM6A* mutations in KS, we examined this gene's 29 coding exons along with its exon–intron boundaries (NM_021140.2) in 32 KS individuals with no *MLL2* mutation, using high-resolution melting analysis combined with direct sequencing. We identified three mutations: c.3717G>A (p.Trp1239*) in patient 1 (male, hemizygous), c.1555C>T (p.Arg519*) in patient 2 (male, hemizygous), and c.3354_3356delTCT (p.Leu1119del) in patient 3 (female, heterozygous) (Fig. 1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM_021140.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. One mutation (c.3354_3356delTCT) occurred de novo; parental samples were unavailable for the other two. Because the two nonsense mutations were outside of the last

Additional Supporting Information may be found in the online version of this article.

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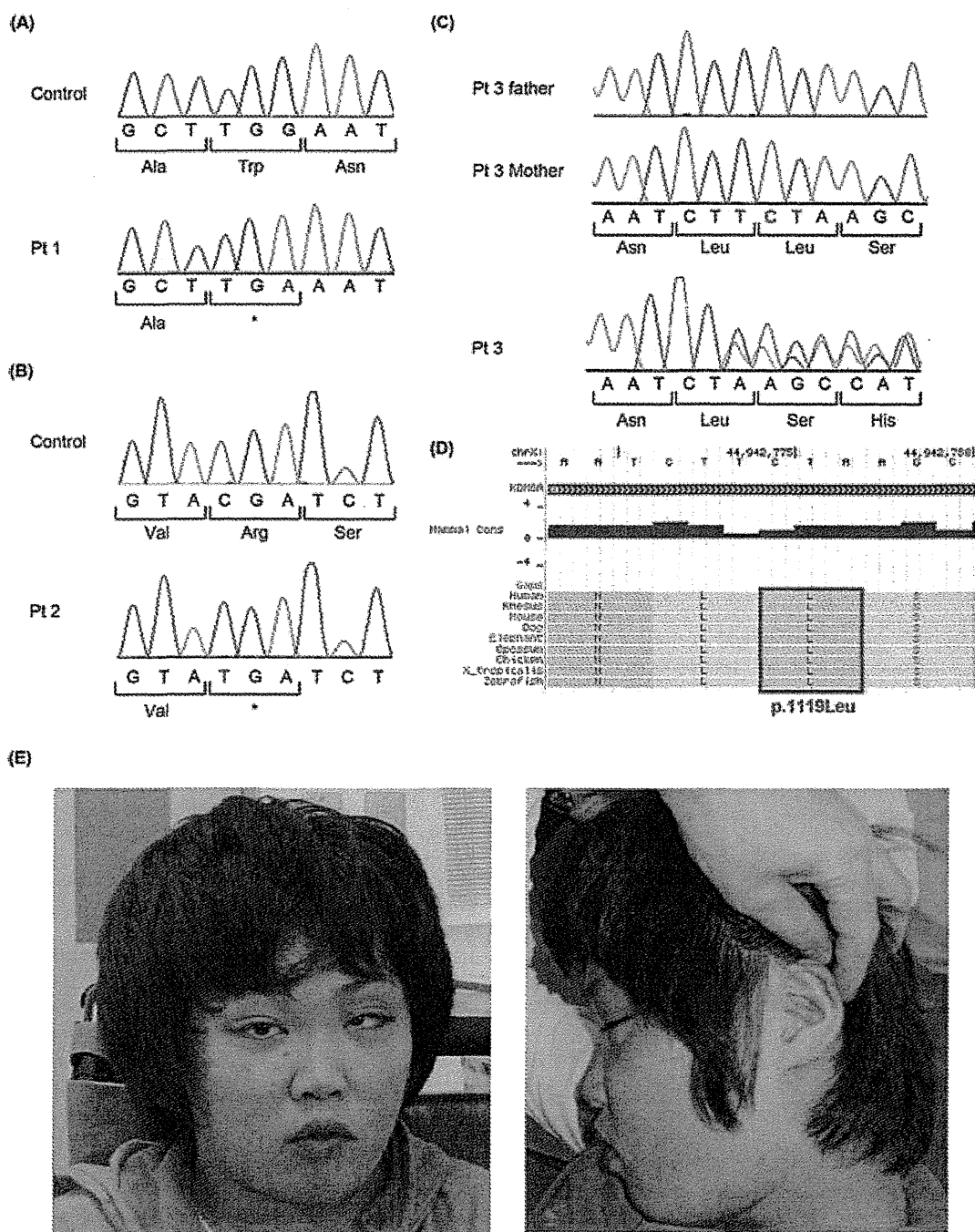


Figure 1. *KDM6A* mutations in three Kabuki syndrome patients. **A–C:** Electropherogram of patient 1: c.3717G>A (p.Trp1239*) (**A**), patient 2: c.1555C>T (p.Arg519*) (**B**), and patient 3: c.3354_3356delTCT (p.Leu1119del) (**C**). Hemizygous changes (**A** and **B**) and a heterozygous change (**C**) can be seen. The altered or deleted nucleotides are written in red. **D:** p. Leu1119 is evolutionarily conserved from zebrafish to human. The position of p.Leu1119 is boxed in red. **E:** Facial photographs of patient 3.

coding exon, and in an exon 55 bp from the 3' most exon–exon junction, the mutant alleles could be subjected to nonsense-mediated mRNA decay (unfortunately living cells from the patients were unavailable, so we could not test this hypothesis). c.3354_3356delTCT in patient 3 would lead to deletion of one amino acid within the functionally important catalytic Jumonji C (JmjC) domain [Lee et al., 2007]. The amino residue p.Leu1119 is evolutionarily conserved from zebrafish to human (Fig. 1D) and plays an important

role in hydrophobic core formation with p.Ile1126 and p.Met1129 to stabilize the JmjC domain [Sengoku and Yokoyama, 2011]. This amino acid deletion may impair helix formation around the mutated residue, resulting in domain destabilization.

Basically, *KDM6A/Kdm6a* escapes X-inactivation in humans and mice [Greenfield et al., 1998; Xu et al., 2008]. However, its expression from the inactive X chromosome is lower (15–35%) than that from the active X chromosome in female mice; thus, *Kdm6a* expression

Table 1. Clinical Features of Patients with a *KDM6A* Mutation

	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Female
Mutation	c.3717G>A	c.1555C>T	c.3354_3356delTCT
Protein change	p.Trp1239*	p.Arg519*	p.Leu1119del
De novo status	NA	NA	De novo
Paternal age at birth	34	42	27
Maternal age at birth	33	40	26
Characteristic face	+	+	+
Microcephaly	+	+	-
Long palpebral fissures	+	+	+
Epicanthus	+	-	-
Lower palpebral eversion	+	+	+
Prominent ear	+	+	-
Auricular deformity	+	+	-
Depressed nasal tip	+	+	NA
Short nasal septum	+	+	NA
Abnormal dentition	+	+	-
Hypodontia	+	+	-
High-arched palate	+	+	-
Micrognathia	+	-	-
Short fifth finger	+	-	+
Developmental delay	+ (Severe)	+ (Severe)	+ (Severe)
Intellectual disability	+ (Severe)	+ (Severe)	+ (Severe)
Short stature	+	+	+
Prenatal growth retardation	+ (-1.96 SD)	+	-
Postnatal growth retardation	+	+	+
Cardiovascular abnormality	+	-	-
Joint laxity	+	+	-
Recurrent otitis media	+	-	-
Deafness	+ ^a	-	NA
Karyotype	46,XY	46,XY	46,XX

^aThe deafness in patient 1 is conductive because of recurrent otitis media. *KDM6A* gene variants were deposited in a gene-specific database (<http://www.lovd.nl/KDM6A>). NA, not analyzed.

in female mice was not twice that in male mice [Xu et al., 2008]. In addition, *UTY* (Yq11.221), a paralog of *KDM6*, has been suspected to partially compensate in males while its function is not well known [Lederer et al., 2012; Xu et al., 2008]. Patient 3 in our study showed a random pattern of X-inactivation with the ratio 57:43 in genomic DNA of peripheral leukocytes. Interestingly, marked skewing of X-inactivation was observed in two female patients reported by Lederer et al. (2012). In their lymphoblast, *KDM6A* deletion was recognized at inactive X chromosome in all 70 mitoses. Here, we propose the threshold model for the pathogenicity of *KDM6A* abnormality (Supp. Fig. S1). The two female patients with a *KDM6A* deletion might not attain the appropriate level of *KDM6A* expression allowing normal development due to existence of specific cells with unfavorable inactivation, whereas male and pure Turner syndrome female with appropriate *KDM6A* expression do not show KS phenotype under assumption of unknown partial functional compensation of *KDM6A* by *UTY* in Y chromosome (only for male) (Supp. Fig. S1).

We reviewed the clinical details of the three patients (Table 1; Supp. Text). All patients were born to unrelated healthy parents. All the three showed severe developmental delay and intellectual disability. Interestingly, patient 3 (female) presented less dysmorphic features and the two male patients 1 and 2 showed a much more severe phenotype with multiple organ involvement (Table 1; Fig. 1E). Null expression of *KDM6A* in males and residual *KDM6A* expression from active X chromosome may explain sex-biased severity (Supp. Fig. S1). Alternatively, it could be explained by a lesser effect of the in-frame mutation in female patient. However, in a previous study, the severity of clinical symptoms varied also among two female patients and a male with a *KDM6A* deletion [Lederer

et al., 2012]. More studies of KS patients with *KDM6A* abnormality are necessary. It is likely that the mutation type as well as the X-inactivation pattern in affected organs in females may determine the severity of KS.

In conclusion, we have described the first three point mutations of *KDM6A* in KS. Our three patients out of 32 *MLL2*-negative patients (mutation detection rate: 9.3%) are comparable to the three patients out of 22 *MLL2*-negative patients (13.6%) previously described [Lederer et al., 2012], regardless of the mutation type. The mutation detection rates for *MLL2* (55–80%) plus *KDM6A* (9–13%) in KS suggest that other gene(s) may be found. Because both *MLL2* and *KDM6A* are histone modifiers, the other pathogenic genes might have related functions. Further research is needed to understand the pathomechanisms of KS as well as the role of histone modification in human disease.

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References

- Banka S, Veeramachaneni R, Reardon W, Howard E, Bunstone S, Ragge N, Parker MJ, Crow YJ, Kerr B, Kingston H, Metcalfe K, Chandler K, et al. 2012. How genetically heterogeneous is Kabuki syndrome? *MLL2* testing in 116 patients, review and analyses of mutation and phenotypic spectrum. *Eur J Hum Genet* 20:381–388.
- Bianca S, Barrano B, Cataliotti A, Indaco L, Ingegnesi C, Ettore G. 2009. Kabuki syndrome and sex chromosomal anomalies: is it really an association? *Fertil Steril* 91:e6.
- Dennis NR, Collins AL, Crolla JA, Cockwell AE, Fisher AM, Jacobs PA. 1993. Three patients with ring (X) chromosomes and a severe phenotype. *J Med Genet* 30:482–486.
- Greenfield A, Carrel L, Pennisi D, Philippe C, Quaderi N, Siggers P, Steiner K, Tam PP, Monaco AP, Willard HF, Koopman P. 1998. The *UTX* gene escapes X inactivation in mice and humans. *Hum Mol Genet* 7:737–742.
- Kuroki Y, Suzuki Y, Chyo H, Hata A, Matsui I. 1981. A new malformation syndrome of long palpebral fissures, large ears, depressed nasal tip, and skeletal anomalies associated with postnatal dwarfism and mental retardation. *J Pediatr* 99:570–573.
- Lederer D, Grisart B, Digilio MC, Benoit V, Crespin M, Ghariani SC, Maystadt I, Dalapiccola B, Verellen-Dumoulin C. 2012. Deletion of *KDM6A*, a histone demethylase interacting with *MLL2*, in three patients with Kabuki syndrome. *Am J Hum Genet* 90:119–124.
- Lee MG, Villa R, Trojer P, Norman J, Yan KP, Reinberg D, Di Croce L, Shiekhat-tar R. 2007. Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. *Science* 318:447–450.
- Matsumoto N, Niikawa N. 2003. Kabuki make-up syndrome: a review. *Am J Med Genet* 117C:57–65.
- McGinniss MJ, Brown DH, Burke LW, Mascarello JT, Jones MC. 1997. Ring chromosome X in a child with manifestations of Kabuki syndrome. *Am J Med Genet* 70:37–42.
- Niikawa N, Kuroki Y, Kajii T, Matsuura N, Ishikiriyama S, Tonoki H, Ishikawa N, Yamada Y, Fujita M, Umemoto H, Iwama Y, Kondoh I, et al. 1988. Kabuki make-up (Niikawa–Kuroki) syndrome: a study of 62 patients. *Am J Med Genet* 31:565–589.
- Niikawa N, Matsuura N, Fukushima Y, Ohsawa T, Kajii T. 1981. Kabuki make-up syndrome: a syndrome of mental retardation, unusual facies, large and protruding ears, and postnatal growth deficiency. *J Pediatr* 99:565–569.
- Rodriguez L, Diego-Alvarez D, Lorda-Sanchez I, Gallardo FL, Martinez-Fernandez ML, Arroyo-Munoz ME, Martinez-Frias ML. 2008. A small and active ring X chromosome in a female with features of Kabuki syndrome. *Am J Med Genet* 146A:2816–21.
- Sengoku T, Yokoyama S. 2011. Structural basis for histone H3 Lys 27 demethylation by *UTX/KDM6A*. *Genes Dev* 25:2266–2277.
- Stankiewicz P, Thiele H, Giannakudis I, Schlicker M, Baldermann C, Kruger A, Dorr S, Starke H, Hansmann I. 2001. Kabuki syndrome-like features associated with a small ring chromosome X and *XIST* gene expression. *Am J Med Genet* 102:286–292.
- Wellesley DG, Slaney S. 1994. Kabuki make-up and Turner syndromes in the same patient. *Clin Dysmorphol* 3:297–300.
- Xu J, Deng X, Watkins R, Distechi CM. 2008. Sex-specific differences in expression of histone demethylases *Utx* and *Uty* in mouse brain and neurons. *J Neurosci* 28:4521–4527.

