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Kabuki syndrome is a congenital anomaly syndrome characterized by developmental delay, intellectual disability, specific facial features including long palpebral fissures and ectropion of the lateral third of the lower eyelids, prominent digit pads, and skeletal and visceral abnormalities. Mutations in *MLL2* and *KDM6A* cause Kabuki syndrome. We screened 81 individuals with Kabuki syndrome for mutations in these genes by conventional methods ($n = 58$) and/or targeted resequencing ($n = 45$) or whole exome sequencing ($n = 5$). We identified a mutation in *MLL2* or *KDM6A* in 50 (61.7%) and 5 (6.2%) cases, respectively. Thirty-five *MLL2* mutations and two *KDM6A* mutations were novel. Non-protein truncating-type *MLL2* mutations were mainly located around functional domains, while truncating-type mutations were scattered through the entire coding region. The facial features of patients in the *MLL2* truncating-type mutation group were typical based on those of the 10 originally reported patients with Kabuki syndrome; those of the other groups were less typical. High arched eyebrows, short fifth finger, and hypotonia in infancy were more frequent in the *MLL2* mutation group than in the *KDM6A* mutation group. Short stature and postnatal growth retardation were observed in all individuals with *KDM6A* mutations, but in only half of the group with *MLL2* mutations. © 2013 Wiley Periodicals, Inc.

Key words: Kabuki syndrome; *MLL2*; *KDM6A*; mutation; genotype–phenotype correlation

INTRODUCTION

Kabuki syndrome (KS; OMIM 147920) is a multiple congenital anomaly syndrome that was originally reported by Niikawa et al. [1981] and Kuroki et al. [1981] (also known as Kabuki make-up syndrome or Niikawa–Kuroki syndrome). KS is diagnosed clinically by characteristic facial features, including long palpebral fissures and ectropion of the lateral third of the lower eyelids, postnatal growth impairment (short stature), developmental delay, intellectual disability, dermatoglyphic abnormalities, visceral and skeletal abnormalities, and immunological dysfunction. The prevalence of the disorder is estimated to be 1 in 32,000 live births [Niikawa et al., 1988]. Two genes have shown to be mutated in patients with KS: *MLL2* (myeloid/lymphoid or mixed-lineage leukemia 2; NM_003482.3) at 12q13.12 and *KDM6A* (lysine (K)-specific demethylase 6A; NM_021140.2) at Xp11.3 [Ng et al., 2010; Lederer et al., 2012; Miyake et al., 2013]. *MLL2* encodes a histone H3 lysine 4 (H3K4)-specific methyl transferase and *KDM6A* is a specific demethylase of histone H3 lysine 27 (H3K27) [Prasad et al., 1997; Lee et al., 2007]. They are both trithorax group proteins and bind each other [Schuettengruber et al., 2007]. These proteins are important for the chromatin state and transcription activation: *MLL2* methylates H3K4 and *KDM6A* removes the H3K27 trimethylation repressive mark

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[Dubuc et al., 2013]. The loss of *MLL2* or *KDM6A* function may lead to repressed transcription [Dubuc et al., 2013].

To our knowledge, there has been no comprehensive screen for mutations in these two genes in the same patient series. In this report, we performed a mutation screen of both genes in 81 patients with KS. We then evaluated the clinical features based on the genetic information.

MATERIALS AND METHODS

Samples

Eighty-one individuals clinically suspected to have KS were incorporated in this study: 77 Japanese, two Caucasians, one Belgian, and one Thai. They were all sporadic except for KMS-79, who had an affected sibling. Peripheral blood samples or saliva samples from the

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patients and their parents (when available) were collected with informed consent and DNA was extracted using a QuickGene-610L (Fujifilm, Tokyo, Japan) or Oragene-DNA kit (DNA Genotek, Inc., Ottawa, Canada) according to the manufacturer's instructions. This study included four previously reported patients (KMS-50, KMS-51, KMS-61, and KMS-71) [Tekin et al., 2006; Torii et al., 2009; Ito et al., 2012]. In addition, three patients with a *KDM6A* mutation were previously described as Patients 1, 2, and 3 by Miyake et al. [2013], and are named KMS-31, KMS-37, and KMS-65, respectively, in this report. This study was approved by the Institutional Review Board of Yokohama City University School of Medicine.

Mutation Screening

Fifty-eight patients (KMS-01 to KMS-69) were screened for *MLL2* mutations by the high-resolution melting (HRM) method using a LightCycler 480 System II (Roche Diagnostics, Indianapolis, IN) and subsequent Sanger sequencing. If an HRM curve pattern was different from those of controls, the DNA sample was Sanger sequenced on an ABI 3500xl or 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and the sequences were analyzed using Sequencher software version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). *KDM6A* was analyzed in samples with no *MLL2* mutation using HRM analysis and Sanger sequencing as above ($n = 37$). For male samples, genotyping using spike-in control male genomic DNA (10%) was performed to detect a hemizygous mutation. The latter 23 patients (KMS-70 to KMS-92), as well as 22 patients with no mutation in either gene detected by conventional methods, were analyzed by targeted resequencing as described in the following section. We judged a variant as pathogenic when it was previously reported to cause KS, or novel variant when it was not observed in unaffected parents or in in-house exome data ($n = 977$), dbSNP135, or EVS6500 (Exome Variant Server, NHLBI GO Exome Sequencing Project, Seattle, WA; <http://evs.gs.washington.edu/EVS/>; accessed March 1, 2013). In addition, the missense mutation predicted to be polymorphism by both of two predictions (Polyphen-2: <http://genetics.bwh.harvard.edu/pph2/> [Adzhubei et al., 2010] and MutationTaster: <http://www.mutationtaster.org/> [Schwarz et al., 2010]) was considered to be non-pathogenic. Parentage analysis was conducted for the patients only when the parental samples were available. TaKaRa Ex Taq and TaKaRa LA Taq (both Takara, Tokyo, Japan) were used for amplification. The primer sequences and PCR conditions are available on request. All pathological variants were confirmed by Sanger sequencing. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_003482.3 for *MLL2*, RefSeq NM_021140.2 for *KDM6A*).

Targeted Resequencing of *MLL2* and *KDM6A* by Next-Generation Sequencing

Ion AmpliSeq Custom Panels (Life Technologies, Inc., Grand Island, NY) covering the entire coding region of *MLL2* and *KDM6A* were created via the Ion AmpliSeq Designer v1.2 (<https://ampliseq.com/browse.action>). Libraries were prepared using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Inc.), with 10 ng of genomic DNA for each primer pool (two pools for this

analysis). An Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) and the associated High Sensitivity D1K Screen Tape (Agilent Technologies) were used to check the size distribution and the concentration of the DNA libraries. Emulsion PCR and enrichment steps were carried out using the Ion OneTouch 200 Template Kit v2 (Life Technologies, Inc.). The amplicon libraries were sequenced on an Ion Torrent Personal Genome Machine system using 314 or 316 chips, and bar-coding was applied with an Ion Xpress Barcode Adapters 1–16 Kit (all Life Technologies, Inc.). Torrent Suite 2.2 (Life Technologies, Inc.) was used for mapping, base calling, and variant calling. Sequences were annotated using SeattleSeq Annotation 134 (<http://snp.gs.washington.edu/SeattleSeqAnnotation134/>). All variants were confirmed by Sanger sequencing.

Whole Exome Sequencing by High-Throughput Next-Generation Sequencing

Whole exome sequencing was performed in five individuals (KMS-09, -18, -21, -23, and -61) who had no *MLL2* or *KDM6A* abnormality by HRM analysis. DNA was processed with a Sure-Select Human All Exon V4 kit (Agilent Technologies), sequenced on a HiSeq2000 (Illumina, Inc., San Diego, CA), and analyzed as previously described [Tsurusaki et al., 2013]. Variants in *MLL2* and *KDM6A* were confirmed by Sanger sequencing.

X-Inactivation Assay

X-inactivation analysis was performed as described [Allen et al., 1992] with slight modification. Briefly, genomic DNA (500 ng) was digested with two methylation-sensitive restriction enzymes, *HpaII* and *HhaI* (New England Biolabs, Beverly, MA), and purified by phenol/chloroform extraction and ethanol precipitation. Digested and undigested DNA samples (10 ng) were separately amplified for the (CAG) n polymorphism at the androgen receptor locus. The forward primer was labeled with 5' FAM dye. PCR products were analyzed on an ABI 3500xl Genetic Analyzer using GeneMapper Software Version 4.1 (Applied Biosystems). The assay was independently performed twice.

cDNA Sequencing

Total RNA was extracted from a lymphoblastoid cell line established from KMS-81 (c.1909_1912del in *KDM6A*) using an RNeasy Plus mini kit (Qiagen, Hilden, Germany) with and without cycloheximide treatment (30 μ g/ml) for 4 hr before cell collection. Reverse transcription (RT) was performed using a Superscript III First-Strand synthesis system for RT-PCR (Life Technologies, Inc.). As the mutation was located in exon 17, the region from the exon 15/16 boundary to the exon 17/18 boundary of *KDM6A* was amplified using cDNA-specific primer pairs (sequences available on request) and sequenced by the Sanger method.

Statistical Analysis

The frequencies of clinical features in the two groups were compared by Fisher's exact test. A difference was considered statistically

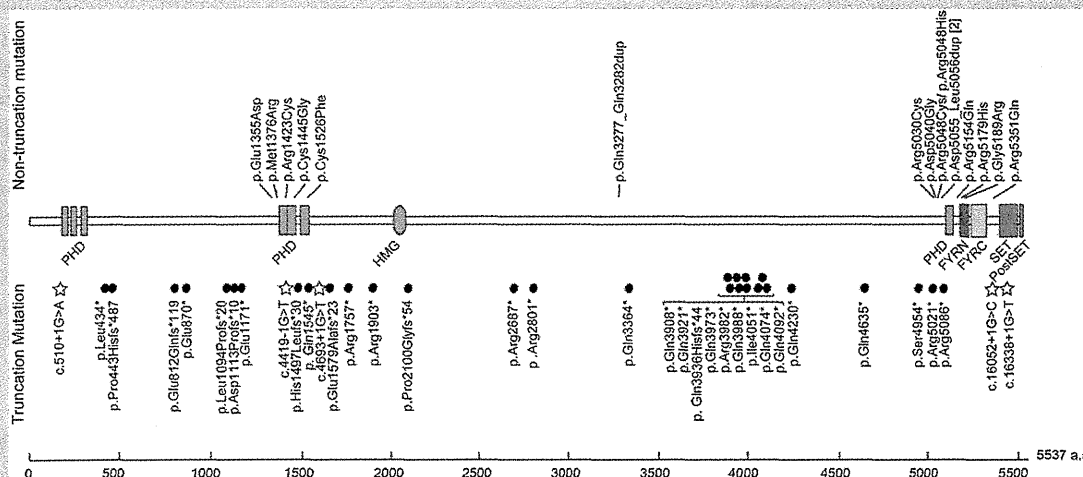


FIG. 1. *MLL2* mutations in patients with Kabuki syndrome. Upper: Non-truncating-type mutations. Middle: *MLL2* protein structure with functional domains. The protein contains seven plant homeodomains (PHD), one high-mobility group (HMG) domain, a Phe-Tyr-rich N-terminal (FYRN) domain, a Phe-Tyr-rich C-terminal (FYRC) domain, a SET (Suvar3-9, Enhancer-of-zeste, Trithorax) domain, and a PostSET domain. These functional domains were based on a prediction by SMART (<http://smart.embl-heidelberg.de/>) and the UniProtKB database (<http://www.uniprot.org/uniprot/014686>). Lower: Truncating-type mutations. Black circles denote nonsense mutations or frameshift mutations. Stars represent splice-site mutations.

significant if $P < 0.05$. Correction for multiple testing was not applied.

RESULTS

Overall Mutation Detection Rates

Pathogenic mutations in *MLL2* and *KDM6A* were found in 50 (61.7%) and five (6.2%) of the 81 patients with KS, respectively (Figs. 1 and 2, Tables I and II). Of the 50 *MLL2* mutations, 35 (70.0%) were predicted to be protein truncating-type and 15 (30.0%) were predicted to be non-truncating-type. Interestingly, non-truncating mutations were mostly localized in or adjacent to the functional domains, while truncating mutations were scattered

throughout the entire coding region (Fig. 1). Fifteen of the *MLL2* mutations have been previously reported (Table I). Three novel variants (not included in the 50 mutations) were considered non-pathogenic (Supplemental Table I). Variant c.10942C > G in patient KMS-22 was predicted to be benign by Polyphen-2 and MutationTaster, c.8813C > T in patient KMS-62 was inherited from an unaffected father, and c.4065A > T in KMS-75 was found heterozygously in our 977 in-house controls. An in-frame duplication in patients KMS-40 and KMS-62, which predicted p.Asp5055_Leu5056dup, was predicted to be polymorphic by MutationTaster, but was previously reported as a pathogenic mutation [Micale et al., 2011]. In addition, the other in-frame mutation in KMS-02 was also predicted to be polymorphic. Unfortunately, parental samples were unavailable for these individuals, except for the mother of patient KMS-62, who did not have this mutation; thus, the de novo status remains unclear. Of the five *KDM6A* mutations including three mutations reported previously [Miyake et al., 2013], four were truncating-type and one was an in-frame deletion located within the Jumonji C domain (Fig. 2).

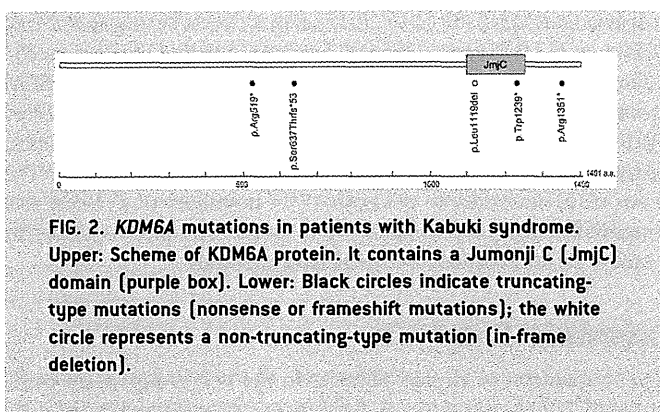


FIG. 2. *KDM6A* mutations in patients with Kabuki syndrome. Upper: Scheme of *KDM6A* protein. It contains a Jumonji C (JmjC) domain (purple box). Lower: Black circles indicate truncating-type mutations [nonsense or frameshift mutations]; the white circle represents a non-truncating-type mutation [in-frame deletion].

Clinical Comparison Between the Mutation-Positive and -Negative Groups

We compared the clinical features of the *MLL2* or *KDM6A* mutation-positive and -negative groups (Supplemental Table II). Long palpebral fissures were observed in almost all patients. Cleft lip/palate was more frequently observed in the mutation-positive group ($P = 0.0197$). Interestingly, developmental delay and intellectual disability were observed in all individuals with mutations but were unobserved in some mutation-negative cases ($P = 0.0314$).

TABLE I. *MLL2* and *KDM6A* Mutations in Patients With KS

Patient ID	Method	Mutation	Predicted amino acid change	De novo	Remarks ^a
Patients with <i>MLL2</i> mutations					
KMS-02	H	c.9831_9848dup	p.Gln3277_Gln3282dup	Unknown	Novel
KMS-08	H	c.12688C > T	p.Gln4230*	Yes	Hannibal et al. [2011]
KMS-13	H	c.2433_2434insCA	p.Glu812Glnfs*119	Yes	Novel
KMS-14	H	c.11806_11807dup	p.Gln3936Hisfs*44	Yes	Novel
KMS-15	H	c.15119A > G	p.Asp5040Gly	Yes	Novel
KMS-17	H	c.5707C > T	p.Arg1903*	Yes	Novel
KMS-18	W	c.12151delA	p.Ile4051*	Yes	Novel
KMS-20	H	c.1300delC	p.Leu434*	Unknown	Novel
KMS-21	W	c.3326_3336dup	p.Asp1113Profs*10	Unknown	Novel
KMS-22	H	c.4127T > G	p.Met1376Arg	Unknown	Novel
KMS-23	W	c.15461G > A	p.Arg5154Gln	Unknown	Li et al. [2011]
KMS-24	H	c.2608 G > T	p.Glu870*	Unknown	Novel
KMS-25	H	c.11917C > T	p.Gln3973*	Unknown	Novel
KMS-27	H	c.15142C > T	p.Arg5048Cys	Yes	Hannibal et al. [2011], Makrythanasis et al. [2013]
KMS-28	H	c.14861C > A	p.Ser4954*	Unknown	Novel
KMS-29	H	c.4419-1G > T	splice site	Unknown	Novel
KMS-30	H	c.4633C > T	p.Gln1545*	Unknown	Novel
KMS-32	H	c.8059C > T	p.Arg2687*	Unknown	Banka et al. [2012b]
KMS-33	T	c.11962C > T	p.Gln3988*	Unknown	Novel
KMS-36	H	c.4736_4737delAG	p.Glu1579Alafs*23	Unknown	Novel
KMS-38	H	c.15143G > A	p.Arg5048His	Unknown	Makrythanasis et al. [2013]
KMS-40	T	c.15163_15168dup	p.Asp5055_Leu5056dup	Unknown	Micale et al. [2011]
KMS-41	H	c.1328delC	p.Pro443Hisfs*487	Yes	Ng et al. [2010]
KMS-42	H	c.16052G > A	p.Arg5351Gln	Yes	Novel
KMS-43	H	c.510 + 1G > A	splice site	Unknown	Novel
KMS-49	T	c.15565G > A	p.Gly5189Arg	Unknown	Novel
KMS-51 ^b	H	c.6297_6298delAC	p.Pro2100Glyfs*54	Yes	Novel
KMS-52	H	c.4693 + 1G > T	splice site	Yes	Novel
KMS-53	H	c.10090C > T	p.Gln3364*	Yes	Novel
KMS-54	T	c.8401C > T	p.Arg2801*	Yes	Novel
KMS-56	H	c.15536G > A	p.Arg5179His	Yes	Ng et al. [2010]
KMS-58	H	c.4333T > G	p.Cys1445Gly	Yes	Novel
KMS-59	H	c.15256C > T	p.Arg5086*	Yes	Banka et al. [2012b]
KMS-60	H	c.11761C > T	p.Gln3921*	Unknown	Novel
KMS-61 ^c	W	c.5269C > T	p.Arg1757*	Yes	Novel
KMS-62	H	c.15163_15168dup	p.Asp5055_Leu5056dup	Unknown ^f	Micale et al. [2011]
KMS-63	T	c.4577G > T	p.Cys1526Phe	Yes	Novel
KMS-69	H	c.11944C > T	p.Arg3982*	Yes	Paulussen et al. [2011]
KMS-70	T	c.13903C > T	p.Gln4635*	Yes	Novel
KMS-71 ^d	T	c.12220C > T	p.Gln4074*	Unknown	Novel
KMS-72	T	c.15061C > T	p.Arg5021*	Yes	Banka et al. [2012b]
KMS-73	T	c.12274C > T	p.Gln4092*	Unknown	Micale et al. [2011]
KMS-76	T	c.4490_4491delAC	p.His1497Leufs*30	Yes	Novel
KMS-78	T	c.16338 + 1G > T	splice site	Yes	Novel
KMS-80	T	c.15088C > T	p.Arg5030Cys	Unknown	Makrythanasis et al. [2013]
KMS-82	T	c.3511G > T	p.Glu1171*	Yes	Novel
KMS-85	T	c.11722C > T	p.Gln3908*	Yes	Paulussen et al. [2011]
KMS-87	T	c.3281_3282delTCT	p.Leu1094Profs*20	Yes	Novel
KMS-88	T	c.16052 + 1G > C	splice site	Unknown	Novel
KMS-91	T	c.4267C > T	p.Arg1423Cys	Unknown	Novel
Patients with <i>KDM6A</i> mutations					
KMS-31 ^e	H	c.3717G > A	p.Trp1239*	Unknown	Miyake et al. [2013]
KMS-37 ^e	H	c.1555C > T	p.Arg519*	Unknown	Miyake et al. [2013]
KMS-65 ^e	H	c.3354_3356delTCT	p.Leu1119del	Yes	Miyake et al. [2013]
KMS-81	T	c.1909_1912delTCTA	p.Ser637Thrfs*53	Yes	Novel
KMS-83	T	c.4051C > T	p.Arg1351*	Unknown	Novel

H, high-resolution melting analysis/Sanger sequencing; T, targeted resequencing; W, whole exome sequencing. RefSeq NM_003482.3 for *MLL2* and RefSeq NM_021140.2 for *KDM6A* were used as reference sequences.

^aReferences are listed when the same mutation has been reported previously.

^bThis patient was reported as proband 1 by Tekin et al. [2006].

^cThe detailed clinical features of this patient were reported by Ito et al. [2013] because of her hypothalamic pituitary complications.

^dThe clinical course of this patient, particularly the idiopathic thrombocytopenic purpura, was reported by Torii et al. [2009].

^eThese patients have been reported in our previous study [Miyake et al., 2013].

^fPatient KMS-62: no mutation in the mother.

TABLE II. *MLL2* Non-Truncating-Type Mutations in Patients With KS

Amino acid change ^a	Patient ID	Domain	Polyphen-2 (score)	MutationTaster
p.Met1376Arg	KMS-22	—	Probably damaging [0.915]	Polymorphism
p.Arg1423Cys	KMS-91	PHD	Probably damaging [1.000]	Disease causing
p.Cys1445Gly	KMS-58	PHD	Probably damaging [1.000]	Disease causing
p.Cys1526Phe	KMS-63	PHD	Probably damaging [0.999]	Disease causing
p.Gln3277 Gln3282dup	KMS-02	—	NA	Polymorphism
p.Arg5030Cys	KMS-80	—	Probably damaging [1.000]	Disease causing
p.Asp5040Gly	KMS-15	—	Probably damaging [1.000]	Disease causing
p.Arg5048Cys	KMS-27	—	Probably damaging [1.000]	Disease causing
p.Arg5048His	KMS-38	—	Probably damaging [1.000]	Disease causing
p.Asp5055 Leu5056dup	KMS-40, 62	—	NA	Polymorphism
p.Arg5154Gln	KMS-23	—	Probably damaging [1.000]	Disease causing
p.Arg5179His	KMS-56	FYRN	Possibly damaging [0.840]	Disease causing
p.Gly5189Arg	KMS-49	FYRN	Probably damaging [1.000]	Disease causing
p.Arg5351Gln	KMS-42	—	Probably damaging [1.000]	Disease causing

^aThe nucleotide mutation nomenclature for these predicted protein mutations are included in Table I.

and $P = 0.1778$, respectively). Blue sclera, lower lip pits, spine/rib abnormality, hip joint dislocation, umbilical hernia, kidney dysfunction, cryptorchidism, liver abnormality, spleen abnormality, premature thelarche, neonatal hyperbilirubinemia, and anemia were observed only in the mutation-positive group.

Clinical Comparison of the *MLL2*-Mutated and *KDM6A*-Mutated Groups

We compared the clinical features between the *MLL2*-mutated and *KDM6A*-mutated groups (Figs. 3–5, Supplemental Table III). High arched eyebrows, short fifth fingers, and hypotonia in infancy were more frequent in individuals with *MLL2* mutations than in individuals with *KDM6A* mutations ($P = 0.0364$, 0.0039 , and 0.0283 , respectively). Short stature was more frequent in individuals with *KDM6A* mutations ($P = 0.0485$). Although not statistically significant, postnatal growth retardation was observed in all individuals with *KDM6A* mutations, whereas this was observed in only half of the individuals with *MLL2* mutations.

Clinical Comparison of Individuals With a *MLL2* Truncating-Type and Non-Truncating-Type Mutation

Most clinical features were observed at a similar ratio in both groups (Supplemental Table IV), except for prominent ears and hypotonia, which were more frequently observed in the truncating-type group than in the non-truncating-type group ($P = 0.0339$ and $P = 0.0248$, respectively). However, the facial appearance of individuals in the truncating-type group was more typical, based on the ten originally reported patients with KS [Kuroki et al., 1981; Nii-kawa et al., 1981], than that in the non-truncating-type group (Figs. 3 and 4). Except for patient KMS-58, the facial appearance of patients with a non-truncating-type mutation was rather less typical. It should be noted that these patients had thick eyebrows (not present in patient KMS-56). Furthermore, ectropion of the

lower eyelid, depressed nasal tip, short columella, and prominent ears all seemed less obvious in the individuals with a non-truncating-type mutation.

X-Inactivation Pattern in Female Patients With a *KDM6A* Mutation

A *KDM6A* mutation was identified in two females (KMS-65 and KMS-81). Individual KMS-65 (c.3354_3356del, which predicts p. Leu1119del) showed a random X-inactivation pattern [Miyake et al., 2013], while individual KMS-81 with a frame-shift mutation showed marked skewing (98:2; Supplemental Fig. 1A). By RT-PCR using mRNA derived from a lymphoblastoid cell line from patient KMS-81, we confirmed that both the mutated and normal alleles were transcribed at similar levels when nonsense-mediated mRNA decay (NMD) was inhibited by cycloheximide treatment (Supplemental Fig. 1B). In untreated cells, or cells treated with dimethyl-sulfoxide (negative control), the mutant allele was transcribed at a lower level than the wild-type allele, indicating that NMD partially eliminated the mutant.

Exome Sequencing

Among the five patients who were also analyzed by whole exome sequencing, mutations were identified and later confirmed by Sanger sequencing in four (Table I). Fragments with an 11 base-pair insertion (c.3326_3336dup) of *MLL2* in patient KMS-21 could not be amplified by Ex Taq, but could be amplified by LA Taq with LA buffer and confirmed by Sanger sequencing. Three other mutations were missed by HRM analysis (Table I).

DISCUSSION

We identified 50 *MLL2* and five *KDM6A* mutations among 81 patients with KS and add to the 246 *MLL2* mutations described in patients with KS [Ng et al., 2010; Hannibal et al., 2011; Li et al., 2011; Micale et al., 2011; Paulussen et al., 2011; Banka et al., 2012a,b;



FIG. 3. Clinical features of patients with Kabuki syndrome harboring a *MLL2* truncating-type mutation. **A:** Facial features of patients with Kabuki syndrome with a *MLL2* truncating-type mutation. The seven panels for patient KMS-54 show serial images at 0, 1, 4, 6.5, 9.5, 22, and 33 years of age, respectively. **B:** Complete right cleft lip/palate and lower lip pits (arrows) in patient KMS-41. **C:** Abnormal dentition in patients KMS-52 and KMS-59. They also showed hypodontia with wide interdentium. **D:** Patient KMS-72 had congenital strabismus and blepharoptosis as well as opacification of the cornea due to Peters anomaly (arrow). **E:** Hand images show short fifth fingers and prominent digit pads (white arrows).



FIG. 4. Clinical features of patients with Kabuki syndrome with a *MLL2* non-truncating-type mutation. A: Facial features of patients with KS and a *MLL2* non-truncating-type mutation. B: Patient KMS-56 showed abnormal dentition [hypodontia with wide interdentium].

Kokitsu-Nakata et al., 2012; Tanaka et al., 2012; Bogershausen and Wollnik, 2013; Makrythanasis et al., 2013] (Human Gene Mutation Database Professional 2012.3; <https://portal.biobase-international.com/hgmd/pro/gene.php>). Our mutation-positivity rate for either gene was 67.9% (55/81), and that for *MLL2* only was 61.7% (50/81); these figures are compatible with those reported in a review (55–80%) [Banka et al., 2012b]. Mutation-negative patients suggest the existence of unknown genes to cause KS or misdiagnosis.

As for the phenotype–genotype relationship, Banka et al. [2012b] suggested that feeding problems, kidney anomalies, premature thelarche, joint dislocation, and palatal malformation were more frequently observed in patients with *MLL2*-mutations than in patients with normal *MLL2* sequence. Hannibal et al. [2011] reported that renal anomalies were more common in patients who had *MLL2* mutations compared to those who did not. Li

et al. [2011] reported that short stature and renal anomalies were more frequent in patients with *MLL2*-mutations than in those with normal *MLL2* sequence. In our study, premature thelarche was observed only in patients with *MLL2* mutations, but this was not significant ($P = 0.1137$). The frequencies of kidney anomalies, hip joint dislocation, and short stature were not different when comparing those with and without *MLL2* mutations ($P = 0.3030$, $P = 1.0000$, and $P = 0.0717$, respectively; Supplemental Table V). High arched eyebrows, palatal malformation (cleft palate/lip), low posterior hairline, and short fifth finger were more frequently observed in individuals with *MLL2* mutations than in patients with normal *MLL2* ($P = 0.0118$, $P = 0.0284$, $P = 0.0493$, and $P = 0.0137$, respectively; Supplemental Table V).

X-inactivation skewing in patients with KS has been discussed since the discovery of the *KDM6A* deletion in a female with KS

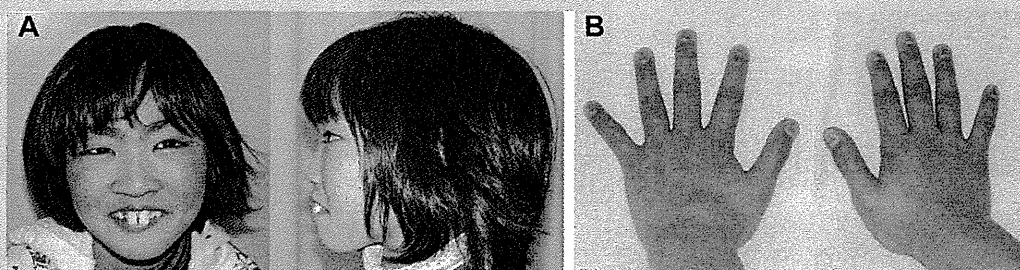


FIG. 5. Clinical features of patients with Kabuki syndrome with a *KDM6A* truncating-type mutation. A: Facial features of patient KMS-81 harboring a *KDM6A* mutation [c.1909_1912del, p.Ser637Thrfs*53]. She showed large front teeth with wide interdentium. B: Hand image of KMS-81. Short fifth finger was not remarkable.

[Lederer et al., 2012; Miyake et al., 2013]. In two female patients reported here, patient KMS-65, who had an in-frame deletion, showed a random X-inactivation pattern, but patient KMS-81, who had a truncating-type mutation, showed marked skewing. X-inactivation skewing was also reported in two affected females with *KDM6A* deletion reported by Lederer et al. [2012]. cDNA sequence analysis of patient KMS-81 indicated that the mutant allele of *KDM6A* was expressed at a similar level to the wild-type allele under NMD inhibition. This result suggests that *KDM6A* mostly escapes X-inactivation in female lymphoblastoid cells. Interestingly, *KDM6A/Kdm6a* escapes X-inactivation in humans and mice, and in mice its expression level from the inactive X chromosome (Xi) was reported as 15–35% of that from the active X chromosome (Xa) [Greenfield et al., 1998; Xu et al., 2008]. We calculated the hypothetical expression assuming a 30% *KDM6A* expression level from Xi and 100% expression from Xa (Supplemental Fig. 2). In patient KMS-81, who showed marked skewing (98:2), either the mutant X chromosome or the wild-type X was inactivated in 98% of cells. If the mutant were inactivated, the expression level would be below 1 ($1.0 \times 0.98 + 0.3 \times 0.02 = 0.986$). If the wild-type were inactivated, the expression level would also be below 1 ($1.0 \times 0.02 + 0.3 \times 0.98 = 0.314$). The KS phenotype is usually unassociated with Turner syndrome (45,X), with the *KDM6A* expression level at 1.0 [Miyake et al., 2013]. It is possible that having a *KDM6A* expression level of 1.0 is essential for a normal human phenotype. Similarly, males with only one copy of *KDM6A* do not manifest KS. We previously mentioned the possibility of *UTY* compensation for *KDM6A* (Supplemental Fig. 2) [Miyake et al., 2013], although human *UTY* lacks demethylase activity [Hong et al., 2007; Lan et al., 2007]. The recent evidence that $X^{Utx-}X^{Utx-}$ homozygous mice demonstrated a more severe phenotype than $X^{Utx-}Y^{Uty+}$ mice indicates that *UTY* can compensate for the loss of *UTX* in embryonic development [Shpargel et al., 2012]. Because mouse and human *UTY* show 75% identity, and 95% identity in the Jumonji C domain [Shpargel et al., 2012], it is likely that normal human males who have only one copy of *KDM6A* are supplemented by *UTY* in a demethylase-independent manner.

Interestingly, $X^{Utx-}Y^{Uty+}$ mice showed small body size [Shpargel et al., 2012]. Similarly, the human *KDM6A*-mutated group exhibited short stature and postnatal growth retardation.

Regarding our mutation detection methods, HRM analysis and Sanger sequencing are both imperfect. Next-generation sequencing is more sensitive (especially for single nucleotide variants and small insertions/deletions), faster, and cheaper due to multiple gene screening and the potential to multiplex. However, a microdeletion involving *MLL2* or *KDM6A* or low-level mosaicism of a single nucleotide variant might be missed by this method. Therefore, in patients who test mutation-negative, more comprehensive approaches might be necessary. In conclusion, we investigated *MLL2* and *KDM6A* mutations and their clinical consequences in patients with KS. The majority of the clinical features were observed at a similar frequency among patients with either *MLL2* or *KDM6A*-mutations. The genetic basis of the patients who tested mutation-negative (20–45%) remains elusive. Further studies are necessary to understand the whole picture of the genetic aspects of KS and its genotype–phenotype relationships.

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De Novo Mutations in *GNAO1*, Encoding a $G\alpha_o$ Subunit of Heterotrimeric G Proteins, Cause Epileptic Encephalopathy

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Heterotrimeric G proteins, composed of α , β , and γ subunits, can transduce a variety of signals from seven-transmembrane-type receptors to intracellular effectors. By whole-exome sequencing and subsequent mutation screening, we identified de novo heterozygous mutations in *GNAO1*, which encodes a $G\alpha_o$ subunit of heterotrimeric G proteins, in four individuals with epileptic encephalopathy. Two of the affected individuals also showed involuntary movements. Somatic mosaicism (approximately 35% to 50% of cells, distributed across multiple cell types, harbored the mutation) was shown in one individual. By mapping the mutation onto three-dimensional models of the $G\alpha$ subunit in three different complexed states, we found that the three mutants (c.521A>G [p.Asp174Gly], c.836T>A [p.Ile279Asn], and c.572_592del [p.Thr191_Phe197del]) are predicted to destabilize the $G\alpha$ subunit fold. A fourth mutant (c.607G>A), in which the Gly203 residue located within the highly conserved switch II region is substituted to Arg, is predicted to impair GTP binding and/or activation of downstream effectors, although the p.Gly203Arg substitution might not interfere with $G\alpha$ binding to G-protein-coupled receptors. Transient-expression experiments suggested that localization to the plasma membrane was variably impaired in the three putatively destabilized mutants. Electrophysiological analysis showed that $G\alpha_o$ -mediated inhibition of calcium currents by norepinephrine tended to be lower in three of the four $G\alpha_o$ mutants. These data suggest that aberrant $G\alpha_o$ signaling can cause multiple neurodevelopmental phenotypes, including epileptic encephalopathy and involuntary movements.

Introduction

Epileptic encephalopathy is a group of neurological disorders characterized by severe and progressive cognitive and behavioral impairments, which are most likely caused or made worse by epileptic activity.¹ Ohtahara syndrome (OS [MIM 308350 and 612164]) is the most severe and the earliest form of epileptic encephalopathy and is characterized by tonic spasms mainly in the neonatal period, seizure intractability, and a suppression-burst pattern on electroencephalography (EEG).² De novo mutations in three genes, *ARX* (MIM 300382), *STXBPI* (MIM 602926), and *KCNQ2* (MIM 602235), have been reported in individuals with OS.^{3–6}

Heterotrimeric guanine-binding proteins (G proteins) are composed of α , β , and γ subunits. In its basal state, $G\alpha$ is bound with guanosine diphosphate (GDP) and forms the $G\alpha\beta\gamma$ complex. When a seven-transmembrane-type receptor binds its agonist, it activates G proteins by cata-

lyzing the exchange of GDP for guanosine triphosphate (GTP) on the $G\alpha$ subunit. Subsequently, GTP-bound $G\alpha$ dissociates from $G\beta\gamma$, and each of the two complexes activates distinct downstream effectors.⁷ In mammals, $G\alpha$ subunits are divided into four classes: $G\alpha_i/o$, $G\alpha_q/11$, $G\alpha_s$, and $G\alpha_{12/13}$.⁷ $G\alpha_o$, encoded by *GNAO1* (MIM 139311), is extremely abundant in brain tissue, where it can constitute up to approximately 0.5% of membrane protein,⁸ suggesting important roles in brain function. In fact, mice lacking $G\alpha_o$ show multiple neurological abnormalities, including generalized tremor, occasional seizures, severe motor-control impairment, hyperalgesia, and behavioral abnormalities with early postnatal lethality.^{9,10}

In this study, de novo *GNAO1* mutations were identified in four epileptic-encephalopathy-affected individuals, three of whom were diagnosed with OS. In addition, two of the four individuals showed involuntary movements, suggesting that aberration of $G\alpha_o$ can cause multiple neurodevelopmental phenotypes.

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