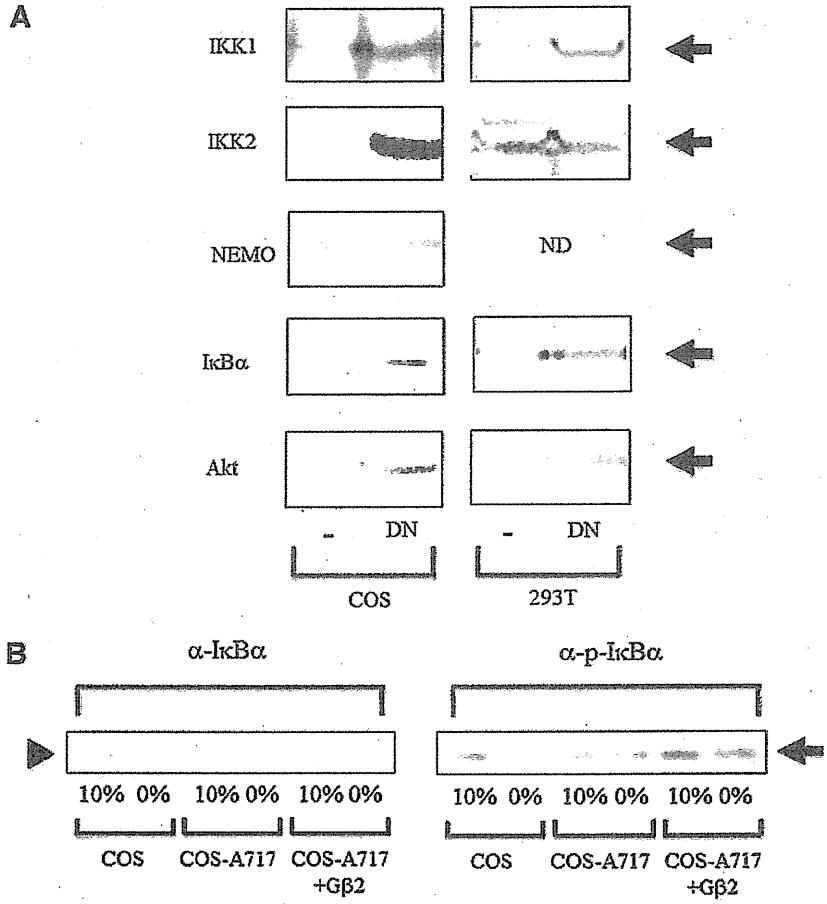

FIG. 5. **(A)** Expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt. COS cells were transfected with 1 μg of the mutant expressing plasmids of IKK1.DN, IKK2.DN, NEMO.DN, superrepressor of IκB (IκB-SR), and Akt.DN (refer to Table 1) in a six-well plate, and the cell lysates were prepared using 100 μL Glo lysis buffer (Promega) after 48h incubation. HEK293T cells were also transfected with the same plasmids, and prepared for Western blot. The description of antibodies is shown in Table 1. The *arrows* indicate the described proteins. N.D: not done. **(B)** COS, COS-A717, and COS-A717-Gβ2 cells were washed with PBS and incubated with (10%) without FBS (0%) for 24 h, and lysed with Glo lysis buffer. The positions of total IκBα, and phospho-IκBα were indicated with *arrowhead* and *arrow*, respectively.

dominant negative mutant is likely to be expressed enough to suppress its endogenous protein.To evaluate the phosphorylation and degradation of IkBα in COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, we carried out Western blotting analysis using their specific antibodies. There were no significant changes in the phosphorylation of IκBα of COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, using a phosphorylation-specific antibody (right panel of Fig. 5B). We could not assess the degradation of IκBα, because the anti-IκBα antibody was actually able to detect the human IκBα protein, but not simian COS IκBα, or because the IκBα expression in COS cell is too low to be detected by this antibody (Fig. 5A and left panel of Fig. 5B). The NF-κB activation by serum deprivation was dependent on IKK1, IKK2, NEMO, and IκBα, and this unique characteristic was not related to the IκBα phosphorylations at S32 and S36.

Because PI3K and Akt are upstream factors of IKKs in the NF-κB activation pathway (Ozes *et al.*, 1999; Romashkova and Makarov, 1999; Xie *et al.*, 2000), we examined whether the Gβ2-induced NF-κB activation occurs through PI3K and Akt activation. However, the PI3K inhibitor,Wortmannin, and a dominant negative mutant of Akt did not affect the Gβ2-induced NF-κB activation in COS-A717 cells (Fig. 4E, F). This result suggests that PI3K and Akt is not involved in the Gβ2-induced NF-κB activation.

Discussion

Many cell stresses activate NF-κB. We have shown here that serum starvation activates NF-κB signal, indicating that serum contains unknown inhibitor(s) of NF-κB signal. Cell stresses, such as radiation (Criswell *et al.*, 2003), oxidation (Marshall *et al.*, 2000), and UV (Kato *et al.*, 2003) positively control the NF-κB signaling. Interestingly, serum negatively regulates the NF-κB signaling, and starvation stress induces NF-κB activation by exclusion of the negative factor of serum.

Serum starvation activated NF-κB signaling in COS cells, but not in COS-A717 cells. The transfection of COS-A717 cells with Gβ2 partially restored the serum starvation-induced NF-κB activation. This result indicates that Gβ2 is required for the starvation-induced NF-κB activation, and the serum inhibitor suppresses the Gβ2-induced signaling pathway (Fig. 6).

Serum starvation of cells is frequently used in many biological experiments, including cell cycle synchronization and induction of apoptosis and autophagy. These biological events induced by starvation unexpectedly include the activation of Gβ2 and NF-κB signals. Therefore, these signaling might affect the synchronization of the cell cycle and the induction of apoptosis and authophagy by starvation, and scientists should consider the effects of the Gβ2 and NF-κB signals in the biological experiments using serum starvation.

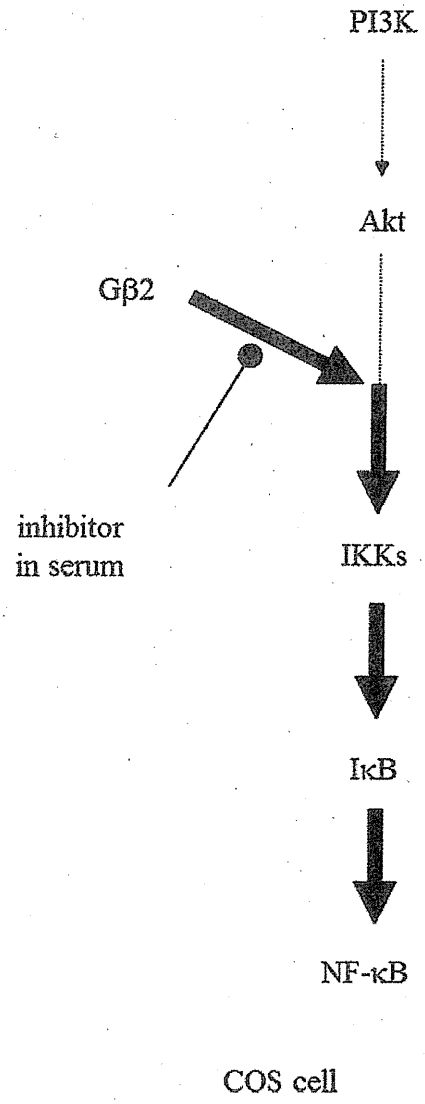

FIG. 6. Signaling pathway of NF-κB activation by Gβ2 or serum starvation.

We are trying to identify the serum inhibitor, and it will provide great impacts into many biological research fields.

NF-κB is constitutively activated in several transformed cell lines, suggesting that NF-κB signaling is involved in cellular transformation. However, the mechanism has not been elucidated yet. COS-A717 cells are mutant cells in which the basal NF-κB activity is much lower compared with the parental COS cells. Here, we showed that COS-A717 cells expressed a lower level of Gβ2 than COS cells, and the transfection of COS-A717 cells with Gβ2 restored the basal NF-κB activity, suggesting that the reduced expression level of Gβ2 is responsible for the defective NF-κB signaling in COS-A717 cells. Furthermore, the knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in the COS cells, but also in the HT1080 cells, another transformed cell line with constitutively activated NF-κB signaling. These results indicate that Gβ2 is required for the constitutive activation of NF-κB in these transformed cells. This conclusion is strongly supported by previous reports showing that certain GPCR signals or the Gβ1γ2 complex activate NF-κB signaling (Xie *et al.*, 2000; Grabiner *et al.*, 2007; Sun *et al.*, 2009). Furthermore, the Tax oncoprotein of HTLV-1 activates NF-κB (Mori *et al.*, 1999; Gohda *et al.*, 2007) as well as the signals of CXCR4, a GPCR, by binding to the Gβ subunit (Twizere *et al.*, 2007), consistent with our conclusion. Although the Gβγ complex activates NF-κB through PI3K (Stephens *et al.*, 1994; Xie *et al.*, 2000), a PI3K inhibitor did not affect the Gβ2-induced NF-κB activation, suggesting that Gβ2 activates independently of PI3K in the NF-κB activation pathway (Fig. 6).

In summary, this study found that Gβ2-induced signaling activates NF-κB independently of PI3K and Akt in COS cells (Fig. 6). Unknown factor(s) present in serum inhibit the Gβ2-induced signaling. Therefore, serum starvation activates NF-κB by removing the serum inhibitor(s). The Gβ2-induced signaling is the target of the serum inhibitor, because exclusion of the serum inhibitor by starvation elevates NF-κB activity in Gβ2-expressing COS cells, but does not affect in Gβ2-defective COS-A717 cells.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Hideki Hayashi, M.D., Ph.D.
Division of Cytokine Signaling
Department of Molecular Microbiology and Immunology
Graduate School of Biomedical Sciences
Nagasaki University
Nagasaki 852-8523
Japan

E-mail: hhayashi@nagasaki-u.ac.jp

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RESEARCH ARTICLE

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MLL2 and *KDM6A* Mutations in Patients With Kabuki Syndrome

Noriko Miyake,[1]* Eriko Koshimizu,[1] Nobuhiko Okamoto,[2] Seiji Mizuno,[3] Tsutomu Ogata,[4] Toshiro Nagai,[5] Tomoki Kosho,[6] Hirofumi Ohashi,[7] Mitsuhiro Kato,[8] Goro Sasaki,[9] Hiroyo Mabe,[10] Yoriko Watanabe,[11] Makoto Yoshino,[11] Toyojiro Matsuishi,[11] Jun-ichi Takanashi,[12] Vorasuk Shotelersuk,[13] Mustafa Tekin,[14] Nobuhiko Ochi,[15] Masaya Kubota,[16] Naoko Ito,[17] Kenji Ihara,[17] Toshiro Hara,[17] Hidefumi Tonoki,[18] Tohru Ohta,[19] Kayoko Saito,[20] Mari Matsuo,[20] Mari Urano,[20] Takashi Enokizono,[21] Astushi Sato,[22] Hiroyuki Tanaka,[23] Atsushi Ogawa,[24] Takako Fujita,[25] Yoko Hiraki,[26] Sachiko Kitanaka,[22] Yoichi Matsubara,[27] Toshio Makita,[28] Masataka Taguri,[29] Mitsuko Nakashima,[1] Yoshinori Tsurusaki,[1] Hirotomo Saitsu,[1] Ko-ichiro Yoshiura,[30] Naomichi Matsumoto,[1]* and Norio Niikawa[19]

[1]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan

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

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

[4]Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu, Japan

[5]Department of Pediatrics, Dokkyo Medical University Koshigaya Hospital, Saitama, Japan

[6]Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto, Japan

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

[8]Department of Pediatrics, Yamagata University Faculty of Medicine, Yamagata, Japan

[9]Department of Pediatrics, Tokyo Dental College Ichikawa General Hospital, Chiba, Japan

[10]Department of Child Development, Kumamoto University Hospital, Kumamoto, Japan

[11]Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan

[12]Department of Pediatrics, Kameda Medical Center, Kamogawa, Chiba, Japan

[13]Center of Excellence for Medical Genetics, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

[14]John P. Hussman Institute for Human Genomics, Miller School of Medicine, University of Miami, Miami, Florida

[15]Department of Pediatrics, Aichi Prefectural Hospital and Habilitation Center for Disabled Children, Dai-ni Aoitori Gakuen, Okazaki, Japan

[16]Division of Neurology, National Center for Child Health and Development, Tokyo, Japan

[17]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

[18]Section of Clinical Genetics, Department of Pediatrics, Tenshi Hospital, Sapporo, Japan

[19]Research Institute of Personalized Health Sciences, Health Science University of Hokkaido, Tobetsu, Japan

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

[21]Department of Pediatrics, University of Tsukuba Hospital, Tsukuba, Japan

[22]Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

[23]Department of Pediatrics, Ohta Nishinouchi Hospital, Tokyo, Japan

[24]Department of Pediatrics, Chikushi Hospital, Fukuoka University, Fukuoka, Japan

[25]Department of Pediatrics, Fukuoka University School of Medicine, Fukuoka, Japan

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

[27]Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan

[28]Education Center, Asahikawa Medical University, Asahikawa, Japan

[29]Department of Biostatistics and Epidemiology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

[30]Department of Human Genetics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

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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

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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 KMD6A removes the H3K27 trimethylation repressive mark [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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*Correspondence to:
Dr. Noriko Miyake or Prof. Naomichi Matsumoto, Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan.
E-mails: nmiyake@yokohama-cu.ac.jp (N. Miyake) or naomat@yokohama-cu.ac.jp (N. Matsumoto)

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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 SureSelect 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, *Hpa*II and *Hha*I (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 μg/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

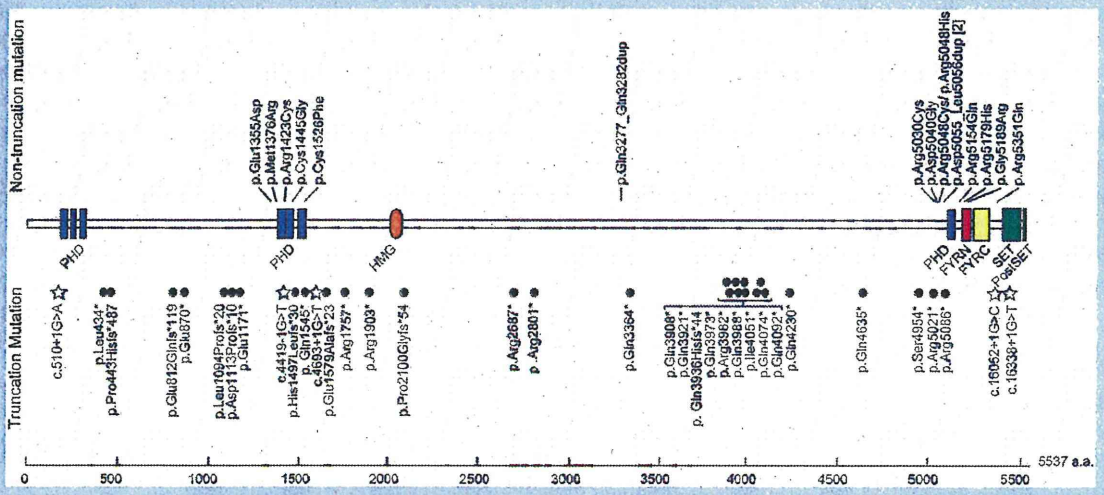

FIG. 1. ***MLL2*** **mutations in patients with Kabuki syndrome. Upper: Non-truncating-type mutations. Middle: MLL2 protein structure with functional domains. The protein contains seven plant homeodomains (PHD), one high-mobility group (HMG) domain, a Phe-Tyr-rich *N*-terminal (FYRN) domain, a Phe-Tyr-rich *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/O14686). 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).

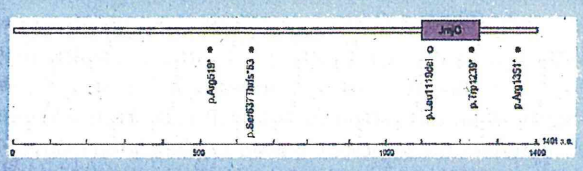

FIG. 2. ***KDM6A*** **mutations in patients with Kabuki syndrome. Upper: Scheme of KDM6A protein. It contains a Jumonji C (JmjC) domain (purple box). Lower: Black circles indicate truncating-type mutations (nonsense or frameshift mutations); the white circle represents a non-truncating-type mutation (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 *MLL2* 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_3282delTC	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 *KDM6A* 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.

[a]References are listed when the same mutation has been reported previously.

[b]This patient was reported as proband 1 by Tekin et al. [2006].

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

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

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

[f]Patient 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

[a]The 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; Niikawa 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 dimethylsulfoxide (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;

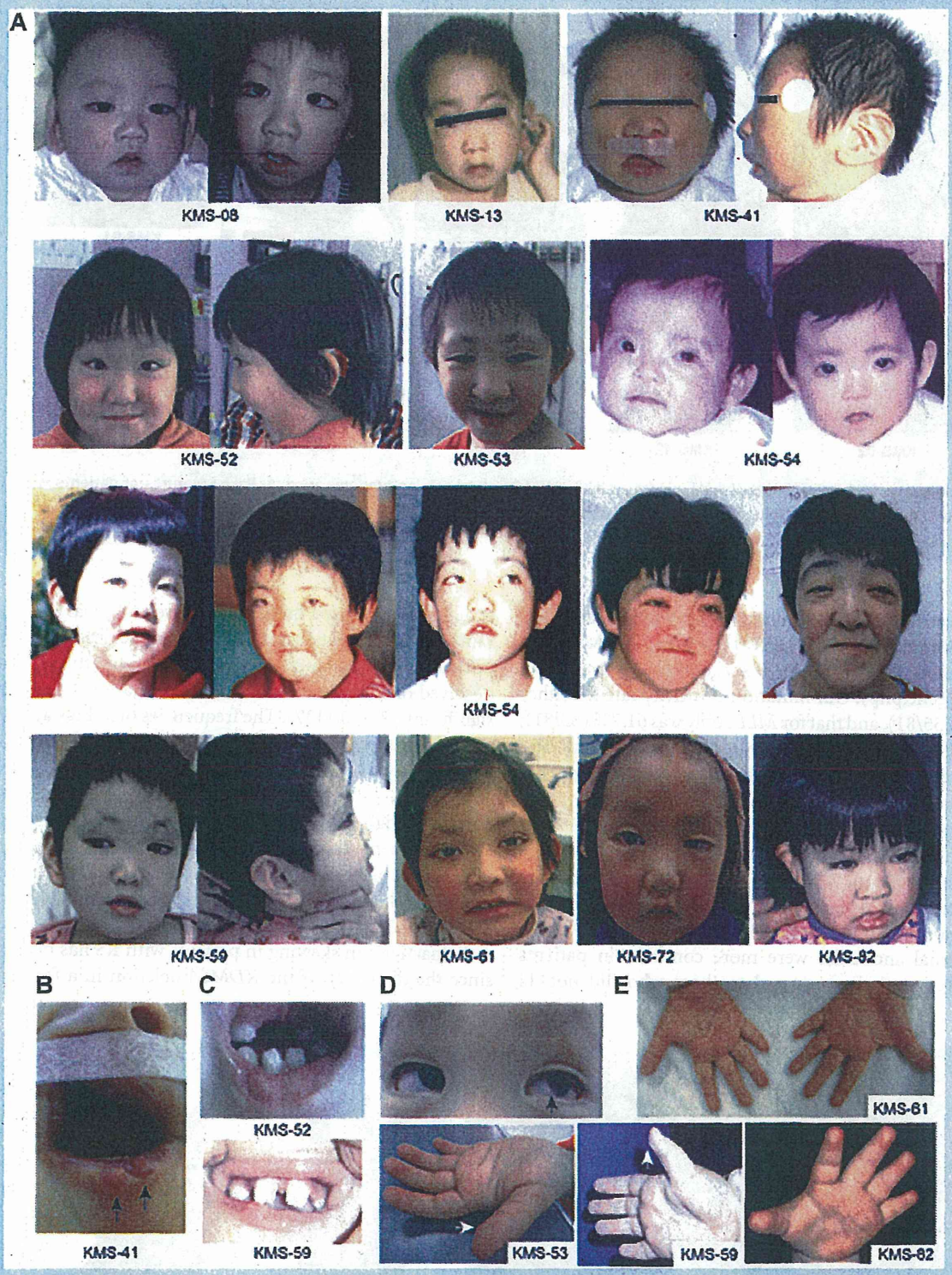

FIG. 3. Clinical features of patients with Kabuki syndrome harboring a MLL2 truncating-type mutation. A: Facial features of patients with Kabuki syndrome with a *MLL2* truncating-type mutation. The seven panels for patient KMS-54 show serial images at 0, 1, 4, 6.5, 9.5, 22, and 33 years of age, respectively. 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).