genome-wide UPD samples in unbiased methylation screens such as bisulphite genome sequencing will aid the identification of additional imprinted loci, which will facilitate study of genetic diseases associated with aberrant imprinting. The general trend until now has been that, while imprinted genes play an important role in fetal development and behavior, evolutionary forces dictated by the genetic conflict have allowed for a lack of conserved imprinting between mouse and humans (54). However, our screen has identified new human-specific imprinted transcripts, all of which have conserved gene orthologues in many taxa. These genes have selected imprinting as a mechanism of transcriptional regulation in humans despite the risk of being functional hemizygous.

MATERIALS AND METHODS

The human reciprocal genome-wide UPD samples

Genomic DNA isolated from three previously described Beckwith–Weidemann syndrome-like cases (16–18) and one Silver–Russell syndrome-like patient (19) was used in this study. Each of these cases had undergone extensive molecular characterization to confirm genome-wide UPD status and level of mosaicism. We used DNA isolated from leukocytes as these samples had minimal mosaicism of a biparental cell line. The genome-wide BWS samples had 9, 11 and 15% biparental contribution, whereas the genome-wide SRS sample had 16%.

Human tissues

Two independent tissue collections were used in this study. All tissues were collected after obtaining informed consent. The Spanish collection was from the Hospital St Joan De Deu tissue cohort (Barcelona, Spain). Normal peripheral blood was collected from adult volunteers aged between 19 and 60 years old. A selection of normal brain samples was obtained from BrainNet Europe/Barcelona Brain Bank. The Japanese tissues were collected at the National Center for Child Health and Development (Tokyo, Japan) and at the Saga University Hospital.

DNA was extracted using either the standard phenol/chloroform extraction method or the QIAamp DNA Blood Midi Kit (Qiagen). RNA was extracted using either Trizol (Invitrogen) or Sepasol®-RNA I Super G (Nacalai Tesque) and cDNA synthesis was carried out as previously described (54). Ethical approval for this study was granted by the Institutional Review Boards at the National Center for Child Health and Development and Saga University and Hospital St Joan De Deu Ethics Committee (Study number 35/07) and IDIBELL (PR006/08).

Cell lines and mouse crosses

Wild-type mouse embryos and placentas were produced by crossing C57BL/6 with *Mus musculus molosinus* (JF1) mice. C57BL/6 (B6) mice were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and JF1/Ms (JF1) mice were obtained from the Genetics Strains Research Center at the National Institute of Genetics, Japan. All animal husbandry and breeding was approved and licensed by the National Research Institute for Child Health and Development, Japan (Approved number A2010–002).

Illumina Infinium methylation27 BeadChip microarray analysis

Approximately 1 μg DNA from the reciprocal genome-wide UPDs, placenta, leukocytes, brain, muscle, fat, buccal cells was subjected to sodium bisulphite treatment and purified using the EZ GOLD methylation kit (ZYMO, Orange, CA, USA). This DNA was then hybridized to the Illumna Infinium Human Methylation27 BeadChip microarray either at the Centro Nacional de Investigaciones Oncologicas (Madrid, Spain) or Genome Science Division, Research Center for Advanced Science and Technology (University of Tokyo, Japan) using Illumina-supplied reagents and protocols. The loci included on this array and the technologies behind the platform have been described previously (55). Before analyzing the methylation data, we excluded possible sources of technical biases that could alter the results. We discarded 109 probes because they had a false-positive rate >0.1. We also excluded 261 probes because of the lack of signal in one of the 11 DNA samples analyzed. Lastly, prior to screening for novel imprinted DMRs, we excluded all X chromosome CpG sites. Therefore, in total we analyzed 26 152 probes in all DNA samples. All hierarchical clustering and β-value evaluation was performed using the Cluster Analysis tool of the BeadStudio software (version 3).

Allelic methylation analysis

A panel of placenta-, leukocyte-, brain- and kidney-derived DNAs were genotyped to identify heterozygous samples. These DNA were converted using the EZ GOLD methylation kit. Approximately 100 ng of converted DNA was used for each bisulphite PCR. Bisulphite-specific primers (Supplementary Material, Table S1) which incorporate the SNPs were used with Hotstar Taq polymerase (Qiagen, West Sussex, UK). Amplifications were performed using either 45 cycles or a nested PCR using 35 cycles for each round. The subsequent PCR products were cloned into pGEM-T Easy vector (Promega) for subsequent sequencing.

Allelic expression analysis

Genotypes on DNA were obtained for exonic SNPs identified in the UCSC browser (NCBI36/hg18, Assembly 2006) by PCR. Sequences were interrogated using Sequencher v4.6 (Gene Codes Corporation, MI) to distinguish informative heterozygote samples. Informative samples were analyzed by RT–PCR. All primers, with the exception of those targeting *FAM50B*, are intron-crossing and incorporated the heterozygous SNP in the resulting amplicon (Supplementary Material, Table S1). RT–PCRs were performed using cycle numbers determined to be within the exponential phase of the PCR, which varied for each gene, but was between 32 and 40 cycles.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Congenital Corneal Staphyloma as a Complication of Kabuki Syndrome

Ryuma Tanaka,[1] Toshiki Takenouchi,[1] Keiko Uchida,[1] Takeshi Sato,[2] Hiroyuki Fukushima,[1] Hiroshi Yoshihashi,[3] Takao Takahashi,[1] Kazuo Tsubota,[4] and Kenjiro Kosaki[1,5]*

[1]Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

[2]Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Kanagawa, Japan

[3]Division of Medical Genetics, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan

[4]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan

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

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Kabuki syndrome has long been clinically defined based mainly on its characteristic eye features. The recent discovery of *MLL2* as a causative gene of Kabuki syndrome has enabled the extreme end of the phenotype to be explored. We herein report on two patients with striking visible congenital staphyloma at birth. A diagnosis of Kabuki syndrome was subsequently made in both patients based on a constellation of characteristic eye features, cardiac abnormalities and severe developmental delay, and finally by the confirmation of *MLL2* mutations. In conclusion, congenital corneal staphyloma is a complication of Kabuki syndrome with *MLL2* mutations. © 2012 Wiley Periodicals, Inc.

Key words: Kabuki syndrome; staphyloma; CHARGE syndrome

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INTRODUCTION

Kabuki syndrome is a congenital syndrome with characteristic facial features reminiscent of the make-up patterns used by Japanese Kabuki actors. Since Kuroki and Niikawa independently described this entity for the first time in 1981 [Kuroki et al., 1981; Niikawa et al., 1981], Kabuki syndrome has become a clinically defined entity, with its diagnosis based mainly on its characteristic facial gestalt. Although Kabuki syndrome was long suspected of being a genetically defined entity, its exact genetic basis remained unknown until 29 years after its initial description, when exome sequencing identified the majority of Kabuki syndrome patients to be caused by mutations in the *MLL2* gene on chromosome 12q12-q14 [Ng et al., 2010]. Now that the genetic basis of Kabuki syndrome has been defined, its phenotypic spectrum should be explored.

Although various ophthalmologic abnormalities other than the typical Kabuki appearance, such as coloboma, had been described in Kabuki syndrome prior to the aforementioned discovery of *MLL2* gene mutations in Kabuki syndrome [Ming et al., 2003], the extreme ends of the ophthalmologic phenotype have not yet been labeled because of the uncertainty of clinical diagnoses based on these unusual features. As an example of an extreme phenotype, we herein present two *MLL2* mutation-positive patients who had congenital corneal staphyloma, a severe form of corneal defect that causes a forward protrusion between the eyelids [Leff et al., 1986; Nelson and Olitsky, 2005].

CLINICAL REPORT

Patient 1

The propositus was a Japanese boy who was prenatally diagnosed as having left pyelectasis and was born at 39 and 4/7 weeks of gestation via vacuum-assisted spontaneous vaginal delivery to a 27-year-old primiparous mother. His birth weight was 3,614 g, and his Apgar scores were 7, 7, and 8 at 1, 5, and 10 min, respectively. At birth, he was noted to have a small but visible structure protruding out of his right eye (Fig. 1). The proband had low-set ears, a high arched

Abbreviations: H3/K4, histone 3 lysine 4.

*Correspondence to:
Kenjiro Kosaki, MD, Center for Medical Genetics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo, 1608582 Japan.
E-mail: kkosaki@z3.keio.jp

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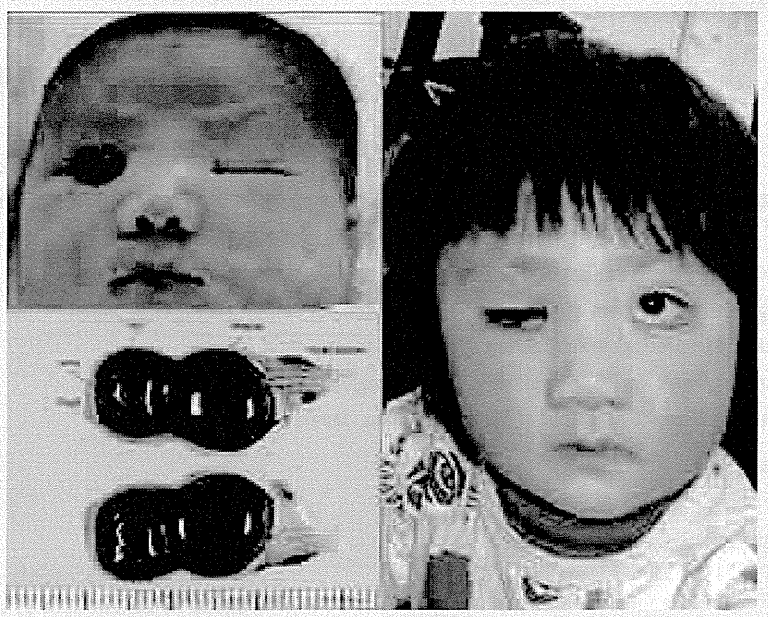

FIG. 1. Facial appearance of Patient 1 at birth (Left Top) and at 4 years of age (Right) and a sagittal section of the surgical specimen of the right eye (Left Bottom).

palate, and a sacral dimple. In addition to a small ventricular septal defect, he was noted to have a left double-renal pelvis, hydronephrosis, ureteropelvic junction stenosis, and right renal malrotation, all of which were managed medically. At 1 month of age, he underwent the total enucleation of his right eye. The pathology report indicated keratoectasia, without evidence of malignancy. His left eye was normal. At age 4 years, he began to ambulate without support but had no meaningful words. A mutation analysis of *MLL2* revealed a truncating mutation in exon 49 (c.15844C>T, p.R5282X).

Patient 2

The second propositus was a non-consanguineous Japanese boy who was born at 39 and 3/7 weeks of gestation via normal spontaneous vaginal delivery. There was no family history of any known inherited condition. His birth weight was 3,379 g, and his height was 50 cm. At birth, bilateral microphthalmia with a right congenital corneal staphyloma were noted (Fig. 2). He underwent a total enucleation of the right eye at the age of 1 month. He had ventricular and atrial septal defects accompanied by symptomatic heart failure, which was treated medically. He has been treated with phenobarbital for epilepsy since the age of 11 months. His motor and mental development were significantly delayed. He first sat at the age of 2 years and 5 months, and he walked independently at the age of 9 years. A clinical diagnosis of Kabuki syndrome was made at the age of 27. A mutation analysis revealed a de novo missense mutation in exon 51 of the *MLL2* gene (c.16294C>T, p.R5432W), confirming the diagnosis of Kabuki syndrome. Currently, he is 30 years old and is unable to communicate verbally; he requires assistance with all his daily activities.

DISCUSSION

Here, we report on two patients with Kabuki syndrome and *MLL2* mutations who had visible congenital corneal staphyloma at birth.

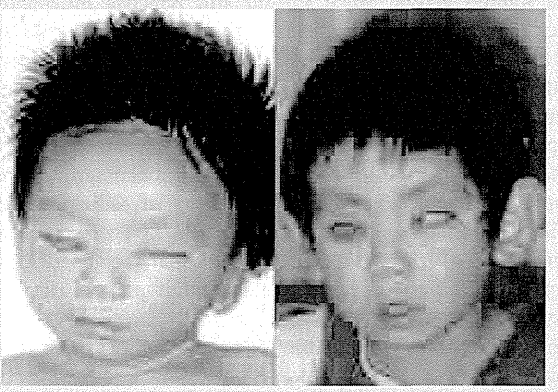

FIG. 2. Facial appearance of Patient 2 at birth (Left) and at 4 years of age (Right).

Since the clinical diagnosis of Kabuki syndrome is heavily dependent on relatively subtle changes in periocular structures (i.e., long palpebral fissures, prominent eyelashes, and eversion of the lateral lower eyelids), a correct diagnosis was delayed because these changes were overshadowed by the striking presence of congenital corneal staphyloma. Our observation of congenital corneal staphyloma in Kabuki syndrome conclusively confirms that congenital corneal staphyloma can have a genetic basis, ending the controversy over its pathogenesis (inflammation versus developmental defect) [Schanzlin et al., 1983].

Staphyloma has been reported in a patient with CHARGE syndrome, another relatively common multiple malformation syndrome that heavily involves facial, especially ophthalmic, structures [Pagon et al., 1981; McMain et al., 2008]. Of note, the cardinal ophthalmic defect in CHARGE syndrome, i.e., coloboma, was also reported in clinically diagnosed Kabuki patients prior to the discovery of *MLL2* as the causative gene [Ming et al., 2003]. Hence, Kabuki syndrome and CHARGE syndrome share coloboma and staphyloma as common ophthalmic developmental defects.

Interestingly, Kabuki syndrome and CHARGE syndrome also share a common defect from a molecular standpoint: the causative genes for both Kabuki syndrome (i.e., *MLL2*) and CHARGE syndrome (i.e., *CHD7*) have histone H3/K4 (H3/K4) methylation as a target for their actions. *MLL2* represents an H3/K4 methyltransferase [Andreu-Vieyra et al., 2010] whereas *CHD7* binding sites contain high levels of H3/K4 mono-methylation [Schnetz et al., 2009]. We speculate that the presence of a common molecular target (i.e., H3/K4) leads to the similar ophthalmic phenotype between Kabuki syndrome and CHARGE syndrome.

In summary, congenital corneal staphyloma was observed in two patients with Kabuki syndrome with *MLL2* mutations. The exact incidence of congenital corneal staphyloma in Kabuki syndrome needs to be investigated in a larger group of patients. Kabuki syndrome should be included in the differential diagnosis of patients with congenital corneal staphyloma as a dysmorphic feature at birth.

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RESEARCH ARTICLE

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Spectrum of *MLL2* (*ALR*) Mutations in 110 Cases of Kabuki Syndrome

Mark C. Hannibal,[1,2] Kati J. Buckingham,[1] Sarah B. Ng,[3] Jeffrey E. Ming,[4] Anita E. Beck,[1,2] Margaret J. McMillin,[2] Heidi I. Gildersleeve,[1] Abigail W. Bigham,[1] Holly K. Tabor,[1,2] Heather C. Mefford,[1,2] Joseph Cook,[1] Koh-ichiro Yoshiura,[5] Tadashi Matsumoto,[5] Naomichi Matsumoto,[6] Noriko Miyake,[6] Hidefumi Tonoki,[7] Kenji Naritomi,[8] Tadashi Kaname,[8] Toshiro Nagai,[9] Hirofumi Ohashi,[10] Kenji Kurosawa,[11] Jia-Woei Hou,[12] Tohru Ohta,[13] Deshung Liang,[14] Akira Sudo,[15] Colleen A. Morris,[16] Siddharth Banka,[17] Graeme C. Black,[17] Jill Clayton-Smith,[17] Deborah A. Nickerson,[3] Elaine H. Zackai,[4] Tamim H. Shaikh,[18] Dian Donnai,[17] Norio Niikawa,[13] Jay Shendure,[3] and Michael J. Bamshad[1,2,3]*

[1]Department of Pediatrics, University of Washington, Seattle, Washington

[2]Seattle Children's Hospital, Seattle, Washington

[3]Department of Genome Sciences, University of Washington, Seattle, Washington

[4]Department of Pediatrics, The Children's Hospital of Philadelphia, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

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

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

[7]Department of Pediatrics, Tenshi Hospital, Sapporo, Japan

[8]Department of Medical Genetics, University of the Ryukyus, Okinawa, Japan

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

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

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

[12]Department of Pediatrics, Chang Gung Children's Hospital, Taoyuan, Taiwan, Republic of China

[13]Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan

[14]National Laboratory of Medical Genetics, Xiangya Hospital, Central South University, Republic of China

[15]Department of Pediatrics, Sapporo City General Hospital, Sapporo, Japan

[16]University of Nevada School of Medicine, Las Vegas, Nevada

[17]Department of Genetic Medicine, Manchester Academic Health Sciences Centre, University of Manchester, England

[18]Department of Pediatrics, University of Colorado, Denver, Colorado

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Mark C. Hannibal, Kati J. Buckingham, and Sarah B. Ng contributed equally to this work.

*Correspondence to:
Michael J. Bamshad, M.D., Department of Pediatrics, University of Washington School of Medicine, Box 356320, 1959 NE Pacific Street, Seattle, WA 98195. E-mail: mbamshad@u.washington.edu

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Kabuki syndrome is a rare, multiple malformation disorder characterized by a distinctive facial appearance, cardiac anomalies, skeletal abnormalities, and mild to moderate intellectual disability. Simplex cases make up the vast majority of the reported cases with Kabuki syndrome, but parent-to-child transmission in more than a half-dozen instances indicates that it is an autosomal dominant disorder. We recently reported that Kabuki syndrome is caused by mutations in *MLL2*, a gene that encodes a Trithorax-group histone methyltransferase, a protein important in the epigenetic control of active chromatin states. Here, we report on the screening of 110 families with Kabuki syndrome. *MLL2* mutations were found in 81/110 (74%) of families. In simplex cases for which DNA was available from both parents, 25 mutations were confirmed to be de novo, while a transmitted *MLL2* mutation was found in two of three familial cases. The majority of variants found to cause Kabuki syndrome were novel nonsense or frameshift mutations that are predicted to result in haploinsufficiency. The clinical characteristics of *MLL2* mutation-positive cases did not differ significantly from *MLL2* mutation-negative cases with the exception that renal anomalies were more common in *MLL2* mutation-positive cases. These results are important for understanding the phenotypic consequences of *MLL2* mutations for individuals and their families as well as for providing a basis for the identification of additional genes for Kabuki syndrome.

Key words: Kabuki syndrome; *MLL2*; *ALR*; Trithorax group histone methyltransferase

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INTRODUCTION

Kabuki syndrome (OMIM#147920) is a rare, multiple malformation disorder characterized by a distinctive facial appearance, cardiac anomalies, skeletal abnormalities, and mild to moderate intellectual disability. It was originally described by Niikawa et al. [1981] and Kuroki et al. [1981] in 1981, and to date, about 400 cases have been reported worldwide [Niikawa et al., 1988; White et al., 2004; Adam and Hudgins, 2005]. The spectrum of abnormalities found in individuals with Kabuki syndrome is diverse, yet virtually all affected persons are reported to have similar facial features consisting of elongated palpebral fissures, eversion of the lateral third of the lower eyelids, and broad, arched eyebrows with lateral sparseness. Additionally, affected individuals commonly have severe feeding problems, failure to thrive in infancy, and height around or below the 3rd centile for age in about half of cases.

We recently reported that a majority of cases of Kabuki syndrome are caused by mutations in *mixed lineage leukemia 2* (*MLL2*; OMIM#602113), also known as either *MLL4* or *ALR* [Ng et al., 2010]. *MLL2* encodes a SET-domain-containing histone methyltransferase important in the epigenetic control of active chromatin states [FitzGerald and Diaz, 1999]. Exome sequencing revealed that 9 of 10 individuals had novel variants in *MLL2* that were predicted to be deleterious. A single individual had no mutation in the protein-coding exons of *MLL2*, though in retrospect, his phenotypic features are somewhat atypical of Kabuki syndrome. In a larger validation cohort screened by Sanger sequencing, we found *MLL2* mutations in approximately two-thirds of 43 Kabuki cases, suggesting that Kabuki syndrome is genetically heterogeneous.

Herein we report on the results of screening *MLL2* for mutations in 110 families with one or more individuals affected with Kabuki syndrome in order to: (1) characterize the spectrum of *MLL2* mutations that cause Kabuki syndrome; (2) determine whether *MLL2* genotype is predictive of phenotype; (3) assess whether the clinical characteristics of *MLL2* mutation-positive cases differ from *MLL2* mutation-negative cases; and (4) delineate the subset of Kabuki cases that are *MLL2* mutation-negative for further gene discovery studies.

MATERIALS AND METHODS

Subjects

Referral for inclusion into the study required a diagnosis of Kabuki syndrome made by a clinical geneticist. From these cases, phenotypic data were collected by review of medical records, phone interviews, and photographs. These data were collected from five different clinical genetics centers in three different countries and over a protracted period of time and forwarded for review to two of the authors (M.B. and M.H.). Data on certain phenotypic characteristics including stature, feeding difficulties, and failure to thrive was not uniformly collected or standardized. Therefore, we decided to be conservative in our analysis and use only phenotypic traits that could be represented by discrete variables (i.e., presence or absence) and for which data were available from at least 70% of cases. In addition, these clinical summaries were de-identified and therefore facial photographs were unavailable from most cases studied. Written consent was obtained for all participants who provided identifiable samples. The Institutional Review Boards of Seattle Children's Hospital and the University of Washington approved all studies. A summary of the clinical characteristics of 53 of these individuals diagnosed with Kabuki syndrome has been reported previously [Ng et al., 2010].

Mutation Analysis

Genomic DNA was extracted using standard protocols. Each of the 54 exons of *MLL2* was amplified using Taq DNA polymerase (Invitrogen, Carlsbad, CA) following manufacturer's recommendations and using primers previously reported [Ng et al., 2010]. PCR products were purified by treatment with exonuclease I (New England Biolabs, Inc., Beverly, MA) and shrimp alkaline phosphatase (USB Corp., Cleveland, OH), and products were sequenced using the dideoxy terminator method on an automated sequencer (ABI 3130xl). The electropherograms of both forward and reverse strands were manually reviewed using CodonCode Aligner (Dedham, MA). Primer sequences and conditions are listed in Supplementary Table I.

For *MLL2* mutation-negative samples, DNA was hybridized to commercially available whole-genome tiling arrays consisting of one million oligonucleotide probes with an average spacing of 2.6 kb throughout the genome (SurePrint G3 Human CGH Microarray 1 × 1 M, Agilent Technologies, Santa Clara, CA). Twenty-one probes on this array covered *MLL2* specifically. Data were analyzed using Genomics Workbench software according to manufacturer's instructions.

RESULTS

All 54 protein-coding exons and intron–exon boundaries of *MLL2* were screened by Sanger sequencing in a cohort of 110 kindreds with Kabuki syndrome. This cohort included 107 simplex cases (including a pair of monozygotic twins) and 3 familial (i.e., parent-offspring) cases putatively diagnosed with Kabuki syndrome. Seventy novel *MLL2* variants that were inferred to be disease-causing were identified in 81/110 (74%) kindreds (Fig. 1 and Supplementary Table II online). These 81 mutations included 37 nonsense mutations (32 different sites and five sites with recurrent mutations), 3 in-frame deletions or duplications (2 different sites and 1 site with a recurrent mutation), 22 frameshifts (22 different sites), 16 missense mutations (11 different sites and 4 sites with recurrent mutations), and 3 splice consensus site (or intron–exon boundary) mutations. None of these variants were found in dbSNP (build 132), the 1000 Genomes Project pilot data, or 190 chromosomes from individuals matched for geographical ancestry. In total, pathogenic variants were found at 70 sites. Additionally, there were 10 sites at which recurrent mutations were observed.

For 25 simplex cases in which we identified *MLL2* mutations, DNA was available from both unaffected parents, and in each case the mutation was confirmed to have arisen de novo (Supplementary Table II online). These included 14 nonsense, 5 frameshift, 3 missense, 2 splice site mutations, and 1 deletion. De novo events were confirmed at 6 of the 10 sites where recurrent mutations were noted. In addition to the 81 kindreds in which we identified causal *MLL2* mutations, we found two *MLL2* variants in each of three simplex cases. In each case, neither *MLL2* mutation could unambiguously

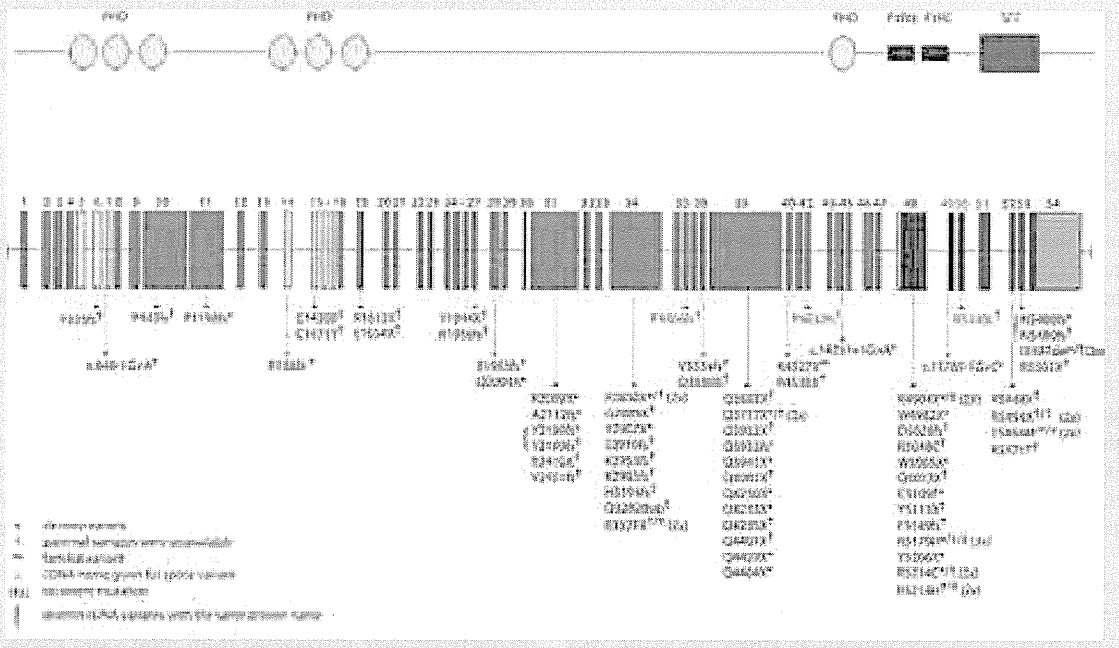

FIG. 1. Genomic structure and allelic spectrum of *MLL2* mutations that cause Kabuki syndrome. *MLL2* is composed of 54 exons that include untranslated regions (orange) and protein coding sequence (blue) including 7 PHD fingers (yellow), FYRN (green), FYRC (green), and a SET domain (red). Arrows indicate the locations of 81 mutations affecting 70 sites found in 110 families with Kabuki syndrome including: 37 nonsense, 22 frameshifts, 16 missense, 3 in-frame deletions/duplications, and 3 splice-site mutations. Asterisks indicate mutations that were confirmed to be de novo and crosses indicate cases for which parental DNA was unavailable. Figure adapted from Ng et al. [2010].

be defined as disease-causing (Supplementary Table II online). In one case, we found both a 21 bp in-frame insertion in exon 39 and a 1 bp insertion in exon 46 predicted to cause a frameshift. However, the unaffected mother also carried the 21 bp insertion suggesting that this is a rare polymorphism, and that the 1 bp deletion is the pathogenic mutation responsible for Kabuki syndrome.

Apparent disease-causing variants were discovered in nearly half (i.e., 22/54) of all protein-coding exons of *MLL2* and in virtually every region known to encode a functional domain (Fig. 1). However, the distribution of variants appeared non-random as 13 and 12 novel variants were identified in exons 48 and 39, respectively. These sites accounted for 25, or more than one-third, of all the novel *MLL2* variants and 31/81 mutations that cause Kabuki syndrome in our cohort. Eleven of the 12 pathogenic variants in exon 39 were nonsense mutations and occurred in regions that encode long polyglutamine tracts.

Four of the families studied herein had two individuals affected with Kabuki syndrome. A pair of monozygous twins with a c.15195G>A nonsense mutation were concordant for mild developmental delay, congenital heart disease, preauricular pits, and palatal abnormalities, but discordant for hearing loss, and a central nervous system malformation. Concordance for mild developmental delay between an affected parent and child was observed in two families with *MLL2* mutations, one with a nonsense mutation, c.13579A>T, p.K4527X, and the other with a missense mutation, c.16391C>T, p.T5464M that was also found in a simplex case. No *MLL2* mutation was found in the remaining affected parent and child pair (Fig. 2).

To examine the relationship between genotype and phenotype, we first compared the frequency of developmental delay, congenital heart disease, cleft lip and/or palate, and structural renal defects between *MLL2* mutation-positive versus *MLL2* mutation-negative cases. No significant difference was observed between groups for three of these four phenotypes (Table Ia). However, renal anomalies were observed in 47% (31/66 cases) of *MLL2* mutation-positive cases compared to 14% (2/14 cases) of *MLL2* mutation-negative cases and this difference was statistically significant ($\chi^2 = 5.1$, df = 1, $P = 0.024$). In 35 cases in two clinical cohorts for whom more complete phenotypic data were available, short stature was observed in 54% (14/26) of *MLL2* mutation-positive cases compared to 33% (3/19 cases) of *MLL2* mutation-negative cases. We also divided the *MLL2* mutation-positive cases into those with nonsense and frameshift mutations and those with missense mutations and compared the frequency of developmental delay, congenital heart disease, cleft lip and/or palate, and structural renal defects between groups. No significant differences were observed between groups (Table Ib).

In 26 independent cases of Kabuki syndrome, including one parent-offspring pair, no *MLL2* mutation was identified. Both persons in the mother–child pair had facial characteristics consistent with Kabuki syndrome (Fig. 2), mild developmental delay, and no major malformations. The mother is of Cambodian ancestry and her daughter is of Cambodian and European American ancestry. In general, most of the *MLL2* mutation-negative Kabuki cases had facial characteristics (Fig. 3) similar to those of the *MLL2* mutation-positive Kabuki cases, and a similar pattern of major malformations (Table I) with the exception of fewer renal abnormalities.

TABLE I. Phenotypic Traits Grouped by *MLL2* Mutation Status (a) and Type (b)

Trait	*MLL2* +	*MLL2* −
Intellectual disability	74/74 (100%)	19/20 (95%)
Mild	51/74 (69%)	10/20 (50%)
Moderate	18/74 (24%)	4/20 (20%)
Severe	4/74 (5%)	3/20 (15%)
Cleft palate, CL/CP	29/72 (40%)	8/18 (44%)
Congenital heart defect	36/71 (51%)	8/19 (42%)
Renal abnormality	31/66 (47%)	2/14 (14%)

Trait	Truncating (N = 59)	Missense (N = 16)
Intellectual disability	54/54 (100%)	15/15 (100%)
Mild	36/54 (67%)	11/15 (73%)
Moderate	13/54 (24%)	4/15 (27%)
Severe	5/54 (9%)	0/15
Cleft palate, CL/CP	23/54 (43%)	3/14 (21%)
Congenital heart defect	30/54 (55%)	4/13 (30%)
Renal anomaly	9/44 (20%)	2/12 (17%)

We screened the *MLL2* mutation-negative cases by aCGH for large deletions or duplications that encompassed *MLL2*. Abnormalities were found in four cases. In one case, a 1.87 kb deletion of chromosome 5 (hg18, chr5:175,493,803–177,361,744) that included *NSD1* and had breakpoints in flanking segmental duplications identical to the microdeletion commonly found in Sotos syndrome, was found. This suggests that this individual has Sotos syndrome, not Kabuki syndrome [Kurotaki et al., 2002]. A second case had a novel 977-kb deletion of chromosome 19q13 (hg18, chr19:61,365,420–62,342,064) encompassing 20 genes. The majority of genes within the deleted region are zinc finger genes, some of which are known to be imprinted in both human and mouse. A third case had a complex translocation t(8;18)(q22;q21). Finally, a fourth case was found to have extra material for the entire chromosome 12. Average log 2 ratio across chromosome 12 was 0.49, most likely representing mosaic aneuploidy of chromosome 12. No aCGH abnormalities were observed in 21 cases and aCGH failed for one case.

DISCUSSION

We have expanded the spectrum of mutations in *MLL2* that cause Kabuki syndrome and explored the relationship between *MLL2* genotype and some of the major, objective phenotypic characteristics of Kabuki syndrome. The majority of variants found to cause Kabuki syndrome are either novel nonsense or frameshift mutations, and appear to arise de novo. While mutations that cause Kabuki syndrome are found throughout the *MLL2* gene, there appear to be at least two exons (39 and 48) in which mutations are identified with a considerably higher frequency. Mutations in these two exons account for nearly half of all mutations found in *MLL2*, while the length of these exons represents ~24% of the *MLL2* open reading frame (ORF). Furthermore, exon 48, the exon in which mutations are most common, comprises only ~7% of the

FIG. 2. Facial photographs of mother and daughter with Kabuki syndrome in whom no causative mutation in *MLL2* was identified. Both have mild developmental delay and no known major malformations.

FIG. 3. Facial photographs of four children diagnosed with Kabuki syndrome in whom no causative mutation in *MLL2* was found. The photograph in the upper left was reprinted from Ng et al. [2010].

MLL2 ORF. Exon 39 contains several regions that encode long polyglutamine tracts suggesting the presence of a mutational hotspot, although no such explanation is obvious for exon 48. A stepwise approach in which these regions are the first screened might be a reasonable approach to diagnostic testing. However, capture of all introns, exons, and nearby *MLL2* regulatory regions followed by next-generation sequencing would be more comprehensive and likely to be less costly over the long term.

Comparison of four of the objective clinical characteristics of *MLL2* mutation-negative versus *MLL2* mutation-positive cases allowed us to explore both the relationship between *MLL2* genotype and Kabuki phenotype and the phenotype of *MLL2* mutation-negative cases. Overall, the clinical characteristics of *MLL2* mutation-positive cases did not differ significantly from *MLL2* mutation-negative cases with the exception that renal anomalies were more common in *MLL2* mutation-positive cases. Similarly, we observed no significant phenotypic—including the severity of developmental delay—differences between individuals grouped by mutation type. However, the phenotypic data available to us for analysis was limited and, for many cases, we lacked specific information about each malformation present. Furthermore, the most typical phenotypic characteristic, the distinctive facial appearance, was not compared in detail between cases although it would be of interest to study facial images "blinded" to mutation status to investigate its power to predict genotype. Analysis of genotype–phenotype relationships using both a larger set of Kabuki cases, and with access to more comprehensive phenotypic information would be valuable.

No *MLL2* mutation could be identified in 26 of the cases referred to us with a diagnosis of Kabuki syndrome. In three of these cases, aCGH identified structural variants that could be of clinical significance although additional investigation is required. A fourth case had the classical deletion observed in individuals with Sotos syndrome, and in retrospect it appears that this case was included in the cohort erroneously. The 22 remaining cases, including 1 parent-offspring pair, represent individuals with fairly classic phenotypic features of Kabuki syndrome without a *MLL2* mutation. This observation suggests that Kabuki syndrome is genetically heterogeneous. To this end, in these 22 cases, we sequenced the protein-coding exons of *UTX*, a gene that encodes a protein that directly interacts with *MLL2* but no pathogenic changes were found (data not shown). Exome sequencing of a subset of these *MLL2* mutation-negative cases to identify other candidate genes for Kabuki syndrome is underway.

Whether Kabuki syndrome is the most appropriate diagnosis for the *MLL2* mutation-negative cases is unclear. Some of the *MLL2* mutation-negative cases appear to have a facial phenotype that differs somewhat from that of the *MLL2* mutation-positive cases. Whether these *MLL2* mutation-negative cases diagnosed by expert clinicians should be considered Kabuki syndrome, a variant thereof, or a separate disorder remains to be determined. Our opinion is that

there is simply not yet enough information to make an informed decision about this issue.

Most of the mutations in *MLL2* are predicted to result in haploinsufficiency. However, it is unclear by what mechanism(s) haploinsufficiency of *MLL2* could cause Kabuki syndrome. *MLL2* encodes a histone 3 lysine 4 (H3K4) methyltransferase, one of at least 10 proteins (genes for which have not to our knowledge yet been screened in Kabuki cases in which *MLL2* mutations were not found) that have been identified to specifically modify the lysine residue at the fourth amino acid position of the histone H3 protein [Kouzarides, 2007]. MLL2 has a SET domain near its C-terminus that is shared by yeast Set1, *Drosophila* Trithorax (TRX) and human MLL1 [FitzGerald and Diaz, 1999]. MLL2 appears to regulate gene transcription and chromatin structure in early development [Prasad et al., 1997]. In mice, loss of *MLL2* results in embryonic lethality before E10.5, and while $Mll2^{+/-}$ mice are viable, they are smaller than wild-type [Ng et al., 2010].

Kabuki syndrome is the most common of a small, but growing group of multiple malformation syndromes accompanied by developmental delay that are caused by mutations in genes that encode proteins involved in histone methylation [De Sario, 2009]. The most notable of these is CHARGE syndrome, which is one of the syndromes often considered in the differential diagnosis of children ultimately diagnosed with Kabuki syndrome. CHARGE syndrome is caused by mutations in *CHD7*, which encodes a chromodomain protein that recognizes the trimethylated H3K4 side chain [Vissers et al., 2004]. Other disorders caused by defects of histone methylation status include several intellectual disability syndromes, some of which are also characterized by malformations (e.g., cleft lip/palate) that overlap with those found in individuals with Kabuki syndrome.

Kabuki syndrome is one of the most common causes of heritable developmental delay. Discovery that mutations in *MLL2* are the most common cause of Kabuki syndrome highlights the role that disrupted regulation of histone methylation plays as a cause of human birth defects. Characterizing the spectrum of mutations in *MLL2* is a small but important first step toward understanding the mechanism(s) that underlies Kabuki syndrome.

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