厚生労働科学研究補助金（難治性疾患克服研究事業）
分担研究報告書

オピッツ三角頭蓋症候群の病態解析に関する研究

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研究要旨

オピッツ三角頭蓋症候群の細胞内病態把握のために、*in vitro* での解析系の確立は重要である。病態解析には罹患組織（細胞）を使用するのが良いが、生体より組織を得ることは困難なため、線維芽細胞等より未分化幹細胞（iPS 細胞）を誘導・樹立し、目的の細胞へ分化させることが必要である。

そこで、線維芽細胞より iPS 細胞の作製を行い、クローンを樹立した。

A. 研究目的

本研究は、奇形症候群の一つオピッツ三角頭蓋症候群の原因とその分子メカニズムを明らかにして発達予後を含めた診断を可能にすることを目的とする。

本研究では、患児で影響を受けるシグナル伝達を解明できる細胞システムを構築するため、患児培養細胞や患児由来 iPS 細胞を樹立する。

B. 研究方法

患者由来の培養細胞より，iPS 細胞の樹立を試みた。

患者由来線維芽細胞へ、ウイルスベクター法を用い、OCT3/4, KLF4, SOX2, c-MYC, NANOG 発現カセットを導入し，フィーダー細胞（マウス胎児性線維芽細胞）上で培養，胚幹細胞様未分化細胞形態を指標として iPS 細胞単離を行った。単離した細胞の幹細胞の同定は，形態およびアルカリフォスファターゼ活性など未分化マーカーの発現を確認して行った。

（倫理面への配慮）

検体の収集は、患者および家族に対し、人権擁護への配慮、不利益・危険性の排除、遺伝カウンセリングなどの詳しい説明を行い、書面により同意を得た後、行った。

C. 研究結果

患者由来の線維芽細胞（一例）より，ウイルスベクター法を用い、OCT3/4, KLF4, SOX2, c-MYC, NANOG 遺伝子発現カセットを導入し，iPS 細胞様形態を示すクローンの

単離ができた。

胚幹細胞様未分化細胞形態を指標として iPS 細胞単離を行い、アルカリフォスファターゼ染色により未分化マーカーの発現を確認した。

D. 考察

疾患細胞での細胞内シグナル伝達の変化や遺伝子発現変化をとらえることは、オピッツ三角頭蓋症候群の病態把握のために重要である。

病態把握のためには症状を呈する組織での発現変化をとらえる必要があるが、iPS 細胞を樹立し、分化誘導により病態組織を再現し、解析することが可能になったと考えられた。

E. 結論

未分化細胞の樹立により、症状を有する細胞への分化誘導というハードルは残っているものの、オピッツ三角頭蓋症候群の分子病態の解明へ一歩近づいた。

F. 研究発表

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16. 宮崎徹：炎症性マクロファージとメタボリックシンドローム、第107回日本内科学会講演シンポジウム、東京、2010年4月9日

G. 知的所有権の取得状況

なし

III 平成２２年度　班員名簿

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IV 研究成果の刊行物に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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成富研二	オピッツ症候群	井村裕夫，辻省次，福井次矢	症候群ハンドブック	中山書店	東京	2011	674
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Hannibal MC, Buckingham KJ, Ng SB, Ming JE, Beck AE, McMillin MJ, Gildersleeve HI, Bigham AW, Tabor HK, Mefford HC, Cook J, Yoshiura KI, Matsumoto T, Matsumoto N, Miyake N, Tonoki H, Naritomi K, Kaname T, Nagai T, Ohashi H, Kurosawa K, Hou JW, Ohta T, Liang D, Sudo A, Morris CA, Banka S, Black GC, Clayton-Smith J, Nickerson DA, Zackai EH, Shaikh TH, Donnai D, Niikawa N, Shendure J, Bamshad MJ.	Spectrum of MLL2 (ALR) mutations in 110 cases of Kabuki syndrome.	American Journal of Medical Genetics	in press		2011
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Kurokawa J, Arai S, Nakashima K, Nagano H, Nishijima A, Miyata K, Ose R, Mori M, Kubota N, Kadowaki Y, Oike Y, T, Koga ., Febbraio M, Iwanaga T & Miyazaki, T.	Macrophage-Derived AIM is endocytosed into adipocytes and decreases lipid droplets via inhibition of fatty acid synthase activity	Cell Metabolism	11 (6)	479-492	2010
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新井郷子、宮崎徹	メタボリック症候群における AIM の機能	病理と臨床	28 (9)	932-939	2010
新井郷子、宮崎徹	メタボリックシンドロームと炎症：脂肪融解タンパク質 AIM の機能	細胞工学	29 (8)	753-758	2010

V　研究成果の刊行物等

オピッツC症候群　Opitz C syndrome

【ICD-10】Q75.0（三角頭蓋）
【OMIM】211750
【特記事項】厚生労働省難治性疾患克服研究事業 研究奨励分野の対象疾患

■**疫学**　発生頻度／800,000～1,000,000人出生に1人
男女比／約1:1

■**遺伝形式**　常染色体劣性または常染色体優性

■**病因と発症に関わる遺伝子**　*CD96* (3q13.3)[1)]

■**診断**　三角頭蓋に加え，瞼裂斜上，内眼角贅皮，鼻根部平低などの特徴的顔貌，歯肉部腫脹を伴う狭高口蓋，多合指趾症，関節拘縮，項部皮膚弛緩，筋緊張低下，発達遅滞などより診断する．心奇形（心房中隔欠損〈ASD〉，心室中隔欠損〈VSD〉，動脈管開存〈PDA〉，Fallot四徴〈TOF〉など），腎奇形（無・低形成など），脳奇形（脳梁欠損・低形成，Dandy-Walker奇形，小脳低形成など）を伴う例がある．また，髄芽腫発症の報告がある．

■**治療**　三角頭蓋に対し，外科的手術が行われることがあるが，発達予後に対する効果については不明．心奇形などの合併奇形に対して外科的治療などを行う．発達遅滞に対しては，療育が中心となる．

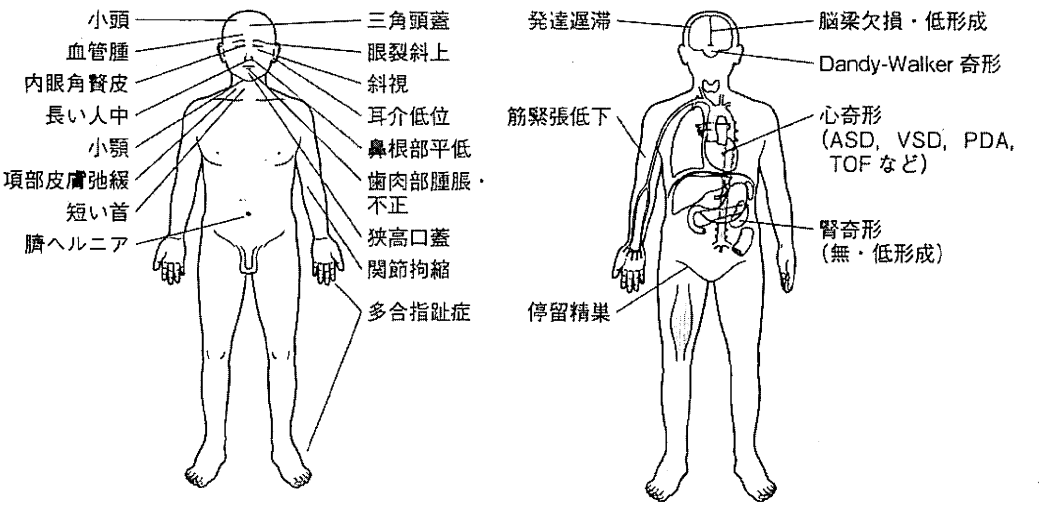
■**関連語・同義語**　オピッツ三角頭蓋症候群，C症候群

■**関連団体・学会**　日本人類遺伝学会／遺伝カウンセリング学会

■**解説**　米国の臨床遺伝医John Marius Opitz（1935年生）が，三角頭蓋に多発奇形を伴う症候群として1969年に初めて報告した．その際，患児のイニシャル（名字）よりC症候群と名付けた[2)]．より症状の重いタイプと思われるBohring・Opitz症候群（子宮内発達遅延なども伴う）は，同じ疾患であるか否か，議論の余地が残されている．

（要　匡）

■**所見**



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オピッツ症候群 Opitz syndrome

■**疫学** 100例以上[3]

■**病因と発症に関わる遺伝子** 常染色体優性型（OMIM 145410） 22q11.2欠失[4]，X連鎖劣性型（OMIM 300000） *Midline 1*（*MID1*）[5]

■**診断** 両眼隔離または内眼角外方偏位，喉頭気管食道裂，口唇裂・口蓋裂および口蓋垂裂，嚥下障害と嗄声，泌尿生殖器奇形（特に男性での尿道下裂と女性での広がった大陰唇），精神遅滞，発達遅滞，および先天性心奇形のある患者で疑う

■**治療** 口唇口蓋裂，尿道下裂，喉頭食道奇形その他の奇形に対して対症療法

■**関連語・同義語** Opitz GBBB症候群，Opitz G症候群，Opitz BBB症候群，尿道下裂–嚥下障害症候群，両眼隔離–尿道下裂症候群，Opitz-Frias症候群

■**解説** 1969年にOpitzが患者家系のイニシャルを使った命名でBBB症候群とG症候群を報告したのに始まる[1,2]．現在はOpitz症候群として統合され，常染色体優性型とX連鎖劣性型に区別される．（成富研二）

■**所見**

一般	誤嚥（A, X） 嚥下障害（X>A） 正常または軽度～中等度の精神遅滞（A, X）
神経	筋緊張低下（A） 弱く粗い喘鳴性泣き声（声）（A）
頭	頭蓋骨非対称性（A）/ 斜頭（20%）
顔	小顎（A） 平坦な人中（A, X） 前頭突出（A, X） 薄い上口唇＋口角下垂（A, X）
眼	両眼隔離 / 内眼角外方偏位（90%）（A, X） 内眼角贅皮＋/－ 副ヒダ（A） 斜視（A）
鼻	幅広く平坦な鼻根（A, X）
口	口唇口蓋裂 / 口蓋垂裂 / 舌裂（A），X（25～35%） 高口蓋（33%）（A, X） 舌小帯短縮（A） 二分口蓋垂
耳	耳介後方回転（A） 伝音性難聴
胸郭	気管食道瘻 肺低形成 / 未分葉肺（A） 裂孔ヘルニア（A）
心臓	（20～25%）PDA, VSD（A, X）
体幹	腹直筋解離（A），臍 / 鼠径ヘルニア（A）
消化器	胆嚢無発生（A） 鎖肛または異所性肛門＋直腸尿道瘻（X>A）
腎臓	腎奇形，尿管奇形（A）
性器	男性：*異常な型の尿道下裂（必発）（A, X） 停留精巣（A, X），二分陰嚢（A） 女性では正常または後部大陰唇の広がり（A）
X線像	脳梁欠損（A, X） 小脳虫部低形成（A） 大脳皮質萎縮（A） 幅広い透明中隔嚢胞（A）
毛髪	富士額（A, X）

（A: 常染色体優性型，X: X連鎖劣性型）

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

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

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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. © 2011 Wiley-Liss, Inc.

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

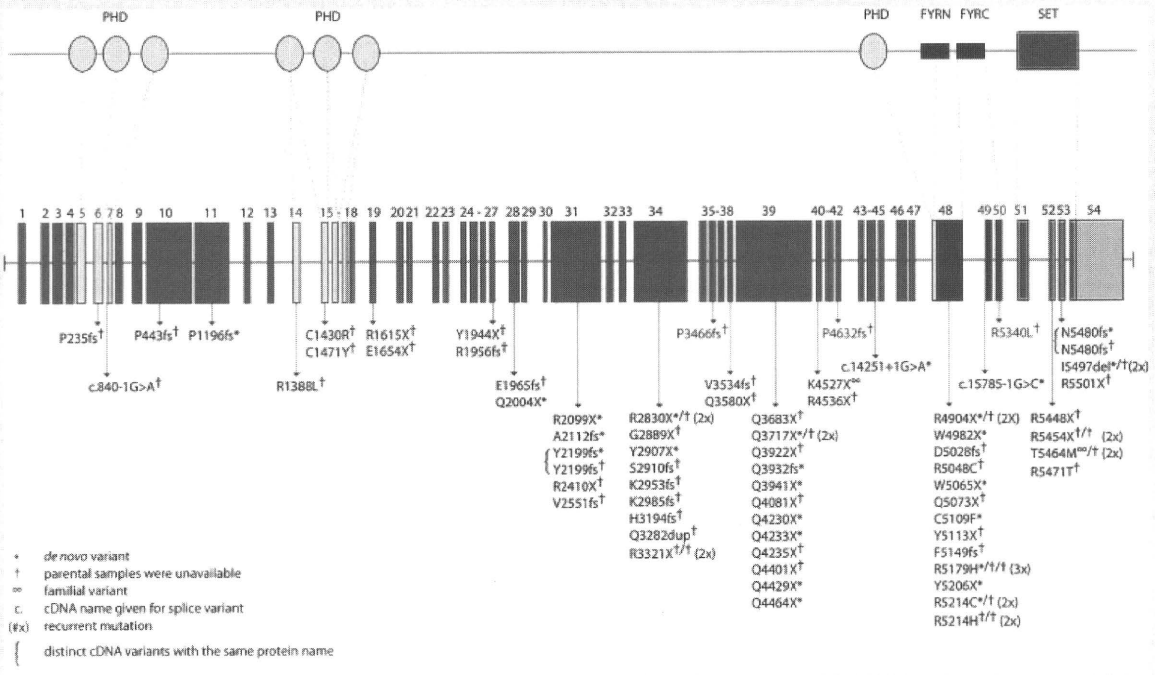

FIG. 1. Genomic structure and allelic spectrum of *MLL2* mutations that cause Kabuki syndrome. *MLL2* is composed of 54 exons that include untranslated regions (orange) and protein coding sequence (blue) including 7 PHD fingers (yellow), FYRN (green), FYRC (green), and a SET domain (red). Arrows indicate 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

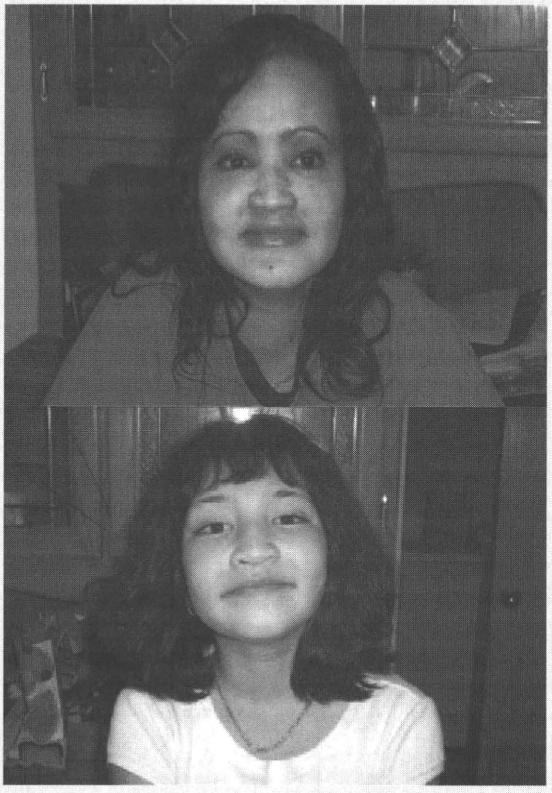

FIG. 2. Facial photographs of mother and daughter with Kabuki syndrome in whom no causative mutation in *MLL2* was identified. Both have mild developmental delay and no known major malformations.

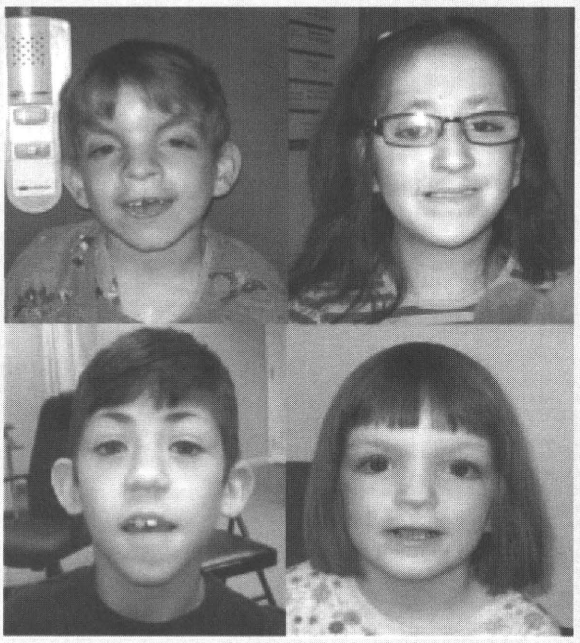

FIG. 3. Facial photographs of four children diagnosed with Kabuki syndrome in whom no causative mutation in *MLL2* was found. The photograph in the upper left was reprinted from Ng et al. [2010].

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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Primer Name	Sequence (5'-3')	Tm (°Celsius)	Amplicon	Size (bp)	Extension Tir (min)
MLL2_Ex1_2_PCR_F1	GATGCCTTCTTCCCAGGATT	60.4	Exons 1 and 2	626	0.5
MLL2_Ex1_2_PCR_R1	TTCCCCAACACTCATTTTCC	59.8			
MLL2_Ex3_5_PCR_F1	GTTTGAGGGCACATGAGGAT	59.9	Exons 3 to 5	1063	1.0
MLL2_Ex3_5_PCR_R1	CCTGGTGCTCACAAAGTTCA	59.9			
MLL2_Ex3_5_Seq_F1	CTGGTGGGCTTCTGAGAGTC	60.0			
MLL2_Ex3_5_Seq_R1	CCTCAGTGTCAGCCAGCTCT	60.8			
MLL2_Ex6_9_PCR_F1	GCAATGTGCTGAGGCTTACA	60.0	Exons 6 to 9	1231	1.5
MLL2_Ex6_9_PCR_R1	ACAGAAAGTGTGGGGTCTGG	60.0			
MLL2_Ex6_9_Seq_F1	CCCTGATTCTGCCCTATTGT	59.7			
MLL2_Ex6_9_Seq_R1	GCATTGGTCAGACAGCAAAG	59.9			
MLL2_Ex10_PCR_F1	CCCTGAAATTCATCCCCTTT	60.1	Exon 10	1715	2.0
MLL2_Ex10_PCR_R1	TGTGCCATGAAGAGTTACAGC	58.9			
MLL2_Ex10_Seq_F1	AAGAGTCACCCCCATCTCCT	59.9			
MLL2_Ex10_Seq_R1	AAATGGTGGGAACAGACGAG	60.0			
MLL2_Ex10_Seq_F2	CCTGAGGACTCACCTGCTTC	60.0			
MLL2_Ex10_Seq_R2	GGACAGATGTGGTCCCTCAG	60.5			
MLL2_Ex11_PCR_F2	GCTGTAACTCTTCATGGCACA	58.9	Exon 11	1463	1.5
MLL2_Ex11_PCR_R2	AGCTCTAGCCCAAACCCATT	60.1			
MLL2_Ex11_Seq_F1	CAGCCTTGGAACCCAGTG	60.2			
MLL2_Ex11_Seq_R1	GCACAGGGGAGCCTTTAAGT	60.6			
MLL2_Ex12_14_PCR_F1	AGTGGGACTCCTGGGCTTAT	60.0	Exons 12 to 14	1552	1.5
MLL2_Ex12_14_PCR_R1	CCACCGTTGAGTTCCAAAGT	60.0			
MLL2_Ex12_14_Seq_F1	TGACTCTGGTCGCAAATCAG	60.0			
MLL2_Ex12_14_Seq_R1	TCCAGTTTTCCCATCTATCCTC	59.4			
MLL2_Ex15_18_PCR_F1	CTGGGGAACAAGAGCAAAAC	59.7	Exons 15 to 18	1049	1.0
MLL2_Ex15_18_PCR_R1	AAGCTAGGGGGTTGGAGCTA	60.2			
MLL2_Ex15_18_Seq_F1	TGACAGAGGCTGGGTTTAGG	60.3			
MLL2_Ex15_18_Seq_R1	CAGAGCTTTAGCACCCAACC	59.9			
				1019	1.0
MLL2_Ex19_21_PCR_F1	GGTTGAAACTTGCAGTTCTGG	59.8	Exons 19 to 21		
MLL2_Ex19_21_PCR_R1	GTCAGACTCGGGTTGAGAGC	60.0			
MLL2_Ex19_21_Seq_F1	AGTGGCTCTGAGGCAAGGTA	60.0			
MLL2_Ex19_21_Seq_R1	TGTCATCCTGCCACTGAGAG	60.0			
MLL2_Ex22_25_PCR_F1	CTCATTGAAAGGGCCAAGAG	59.8	Exons 22 to 25	1161	1.0
MLL2_Ex22_25_PCR_R1	AGGACTCCCCACCAGAGAAG	60.6			
MLL2_Ex22_25_Seq_F1	TGGGAGTGAGTGGTGTGAGA	60.3			
MLL2_Ex22_25_Seq_R1	ATCTGATGCCCAGAACAGGT	59.5			
MLL2_Ex26_27_PCR_F1	CTTCTCTGGTGGGGAGTCCT	60.6	Exons 26 and 27	568	0.5
MLL2_Ex26_27_PCR_R1	CCCAAAAGAGGAGGGTCACT	60.5			
MLL2_Ex28_30_PCR_F1	TCCCCATTCCCTTGTTAGTG	59.8	Exons 28 to 30	910	1.0
MLL2_Ex28_30_PCR_R1	AGACCAGGCATAGGGCAGT	59.7			
MLL2_Ex28_30_Seq_F1	ATGGATTAGCGTGGGAACTG	60.0			
MLL2_Ex28_30_Seq_R1	CACTCCCTACCCAGAAGCAG	59.9			
MLL2_Ex31_PCR_F3*	CCCTAAGGCTGTGTCCCATA	60.0	Exon 31	2193	2.0