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Complete List of Authors:	<p>Paulussen, Aimee; Maastricht University Medical Center (MUMC+), Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology &amp; Developmental Biology (GROW)</p> <p>Stegmann, Alexander; Maastricht University Medical Center (MUMC+), Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology &amp; Developmental Biology (GROW)</p> <p>Blok, Marinus; Maastricht University Medical Center (MUMC+), Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology &amp; Developmental Biology (GROW)</p> <p>Tserpelis, Demis; Maastricht University Medical Center (MUMC+), Clinical Genetics</p> <p>Posma-Velter, Crool; Maastricht University Medical Center (MUMC+), Clinical Genetics</p> <p>Detisch, Yvonne; Maastricht University Medical Center (MUMC+), Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology &amp; Developmental Biology (GROW)</p> <p>Smeets, Eric; Maastricht University Medical Center (MUMC+), Department of Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology &amp; Developmental Biology (GROW)</p> <p>Wagemans, Annemieke; Maastricht University Medical Center (MUMC+), Clinical Genetics</p> <p>Schrander, Jaap; Maastricht University Medical Center (MUMC+), Department of Paediatrics</p> <p>van den Boogaard, Marie-José; University Medical Center Utrecht, Department of Medical Genetics</p> <p>van der Smagt, Jasper; University Medical Center Utrecht, Department of Medical Genetics</p> <p>Haeringen, Arie; Leiden University Medical Center, Department of Human and Clinical Genetics</p> <p>Stolte-Dijkstra, Irene; University Medical Center Groningen, Department of Genetics</p> <p>Kerstjens-Frederikse, Wilhelmina; University Medical Center Groningen, Department of Genetics</p>

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26	Mancini, Grazia; Erasmus University Medical Center, Department of Clinical Genetics Wessels, Marja; Erasmus University Rotterdam, Human Genetics; Erasmus University Medical Center, Department of Clinical Genetics Hennekam, Raoul; University of Amsterdam, Department of Pediatrics, Academic Medical Center Vreeburg, Maaike; Maastricht University Medical Center (MUMC+), Department of Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology & Developmental Biology (GROW) Geraedts, Joep; Maastricht University Medical Center (MUMC+), Department of Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology & Developmental Biology (GROW) Ravel, Thomy; University of Leuven, Center for Human Genetics Fryns, Jean-Pierre; University of Leuven, Center for Human Genetics Devriendt, Koen; University of Leuven, Center for Human Genetics Smeets, H. J. M.; Maastricht University Medical Center (MUMC+), Department of Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology & Developmental Biology (GROW) Schrandt-Stumpel, Constance; Maastricht University Medical Center (MUMC+), Department of Clinical Genetics; Maastricht University Medical Center (MUMC+), School for Oncology & Developmental Biology (GROW)
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Review

# MLL2 mutation spectrum in 45 patients with Kabuki Syndrome



Aimée DC Paulussen<sup>1,3\*</sup>, Alexander PA Stegmann<sup>1,3\*</sup>, Marinus J Blok<sup>1,3</sup>, Demis Tserpelis<sup>1</sup>, Crool Posma-Velter<sup>1</sup>, Yvonne Detisch<sup>1,3</sup>, Eric EJGL Smeets<sup>1,3</sup>, Annemieke Wagemans<sup>1</sup>, Jaap JP Schrande<sup>2</sup>, Marie-José H van den Boogaard<sup>4</sup>, Jasper van der Smagt<sup>4</sup>, Arie van Haeringen<sup>5</sup>, Irene Stolte-Dijkstra<sup>6</sup>, Wilhelmina S Kerstjens-Frederikse<sup>6</sup>, Grazia M Mancini<sup>7</sup>, Marja W Wessels<sup>7</sup>, Raoul CM Hennekam<sup>8</sup>, Maaïke Vreeburg<sup>1,3</sup>, Joep Geraedts<sup>1,3</sup>, Thomy de Ravel<sup>9</sup>, Jean-Pierre Fryns<sup>9</sup>, Hubert J Smeets<sup>1,3</sup>, Koenraad Devriendt<sup>9\*</sup>, and Constance TRM Schrande-Stumpel<sup>1,3\*</sup>

Departments. of <sup>1</sup>Clinical Genetics and <sup>2</sup>Paediatrics, <sup>3</sup>School for Oncology & Developmental Biology (GROW), Maastricht UMC<sup>+</sup>, Maastricht, the Netherlands; <sup>4</sup>Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands; <sup>5</sup>Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, the Netherlands; <sup>6</sup>Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; <sup>7</sup>Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands; <sup>8</sup>Department of Pediatrics, Academic Medical Center, University of Amsterdam, The Netherlands; <sup>9</sup>Center for Human Genetics, University of Leuven, Belgium.

\*These authors contributed equally to this work

\*Correspondence to A.D.C. Paulussen, PhD, A.P.A. Stegmann, PhD, Department of Clinical Genetics, Maastricht University Medical Center, Joseph Bechlaan 113, 6229 GR Maastricht, The Netherlands. E-mail: aimee.paulussen@mumc.nl, sander.stegmann@mumc.nl.

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**ABSTRACT:** Kabuki Syndrome (KS) is a rare syndrome characterized by **intellectual disability** and multiple congenital abnormalities, in particular a distinct dysmorphic facial appearance. KS is caused by mutations in the *MLL2* gene, encoding an H3K4 histone methyl transferase which acts as an epigenetic transcriptional activator during growth and development. Direct sequencing of all 54 exons of the *MLL2* gene in 45 clinically well-defined KS patients identified 34 (75.6%) different mutations. One mutation has been described previously, all others are novel. Clinically, all KS patients were sporadic, and mutations were *de novo* for all 27 families for which both parents were available. We detected nonsense (n=11), frameshift (n=17), splice site (n=4) and missense (n=2) mutations, predicting a high frequency of absent or non-functional *MLL2* protein. Interestingly, both missense mutations located in the C-terminal conserved functional domains of the protein. Phenotypically our study indicated a statistically significant difference in the presence of a distinct facial appearance (p=0.0143) and growth retardation (p=0.0040) when comparing KS patients with an *MLL2* mutation compared to patients without a mutation. Our data **double** the number of *MLL2* mutations in KS reported so far and **widen** the spectrum of *MLL2* mutations and disease mechanisms in KS. ©2010 Wiley-Liss, Inc.

**KEY WORDS:** Kabuki syndrome, *MLL2*, mutation, histone methyl transferase

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## INTRODUCTION

Kabuki syndrome (KS) (or Niikawa-Kuroki syndrome, MIM# 147920) is a rare syndrome, characterized by **intellectual disability** and distinct dysmorphic features, which was originally described by two independent groups in Japan (Kuroki et al., 1981; Niikawa et al., 1981). The estimated prevalence of KS was estimated at 1:32,000 in the Japanese population. Dominant clinical findings include a characteristic facial appearance, developmental delay and growth retardation and additional features such as hypodontia and persistent fetal fingerpads (Adam and Hudgins, 2005; Matsumoto and Niikawa, 2003). The unique facial characteristics observed in KS include elongated palpebral fissures and eversion of the lateral one third of the lower eyelid, an arched or nicked eyebrow with sparseness of hairgrowth in the lateral half, relatively large ears with simplified morphology, a depressed nasal tip and a full lower lip. Recently, a gene causing KS was identified through exome sequencing, reporting mutations in the histone methyl transferase (HMT) gene *MLL2* in 66% of 53 patients with Kabuki syndrome (Ng et al., 2010). *MLL2* is a member of the so called mixed lineage leukemia (MLL) family of HMT proteins, of which *MLL1* (previously called *ALL1*) has been extensively studied because of its frequent involvement in chromosomal rearrangements in acute myeloid and/or lymphatic leukemia (Meyer et al., 2006). *MLL2* is the paralog of *MLL1*, arisen through gene duplication, and is transcribed into a 5,537 amino acid residue protein (NCBI reference sequence NP\_003473.3). The MLL protein family is part of the SET domain containing group of HMTs in humans, which is evolutionary homologous to the *Drosophila* trithorax protein (TRX) (Dillon et al., 2005; Qian and Zhou, 2006). SET domains are highly conserved in evolution and are the essential catalytic protein subunits that execute the histone lysine methylation which is the core function of HMTs. MLL proteins require complex formation with other proteins, which allows recruitment to target genes and facilitates the catalytic function of the protein's SET domain. MLL proteins add methyl groups specifically to the fourth lysine residue of the lysine side chains of histone H3, referred to as H3K4 methylation which is a transcriptional activation mechanism. Histone methylation in general thus constitutes an evolutionary conserved control mechanism for (epigenetic) gene regulation. Multiple MLL containing protein complexes have been identified in non-redundant constellations despite considerable sequence and functional domain similarities among them (Ansari and Mandal, 2010; Eissenberg and Shilatifard, 2010). MLL proteins in general are involved in embryogenesis and development most notably through regulation of HOX gene expression and their interaction with nuclear receptors suggests a role in steroid-hormone controlled signaling. *MLL2* is involved in regulation of cell adhesion, growth impairment and cell motility (Issaeva et al. 2007). Interestingly, methyl transferase pathways in general have been implicated before in the pathology of MR syndromes such as Kleefstra Syndrome (MIM# 610253) which is caused by mutations in the *EHMT1* gene, an H3K9 methyl transferase, and Schinzel-Giedion syndrome (MIM#: 269150) which is caused by mutations in the *SETBP1* gene that encodes a protein known to bind to SET domains (Hoischen et al., 2010; Kleefstra, et al., 2009).

## MATERIALS AND METHODS

### Patient cohort

The patient cohort consists of 45 index patients (31 female, 14 male) with Kabuki syndrome from the Netherlands (n=38) and Belgium (n=7), diagnosed by pediatricians and clinical geneticists. For the current mutation screen, all patient files were reviewed for inclusion by two clinical experts (CSS, YD) **using the guideline: "Management of Kabuki Syndrome, a Clinical Guideline" published online (<http://www.dyscerne.org/dysc/guidelines>)**. In order to **assess** a potential genotype-phenotype correlation detailed photographic evidence of the KS characteristic facial appearance of the patients was evaluated by two independent clinical experts (JS, MV) without prior knowledge of *MLL2* mutation carrier status. A subset of patients have been extensively studied (Schrandt-Stumpel, et al., 2005) and all have undergone routine cytogenetic evaluation showing normal karyotypes at the 550-band resolution levels in the past. The majority of the cohort has also been tested on various SNP-microarray and array-CGH platforms which, with the exception of one patient (Table 1, nr.10) did not result in the identification of any clinically significant *de novo* CNVs (data not shown) in line with reports by others (Kuniba, et al., 2009). Patient 10 has been reported before, because of a *de novo* deletion of exon 5 of the *MACROD2* gene, identified by array-CGH (Maas et al., 2007). Written permission for the studies was obtained from each of the patient's parents or caretakers and the study protocol was approved after evaluation by the local Medical Ethics Committee of the Maastricht University Medical Center (MUMC+).

### PCR and sequence analysis

DNA was extracted from each proband and their parents (if available) from two independent peripheral blood samples following standard procedures. Mutation screening of the complete coding region of the *MLL2* gene was performed by direct sequence analysis in both directions using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Primer sets for all 54 exons spanning the *MLL2* coding region, including intron-exon boundaries, were either designed by Applied Biosystems (AB) for VariantSEQr SNP discovery or were custom designed. All primers were tagged with M13 extensions to customize further sequence-analysis. All PCR's were performed using a PE9700 thermal cycler. Full details of primer sequences, amplicon lengths and PCR conditions are available on request from the authors. Sequences were aligned with the known *MLL2* coding sequence (NCBI reference sequence accession NM\_003482.3). Mutation analysis was performed using Mutation Surveyor V3.30 (©SoftGenetics, PA, USA). Mutations identified were confirmed on a DNA sample extracted from the second independent blood sample from the proband if available. The pathogenicity of the mutations was based on a number of genetic criteria: *de novo* occurrence, predicted non-functionality of mutated proteins, location, type and conservation of mutated amino-acids. In silico prediction of functional consequences was performed using Align GVGD ([http://agvgd.iarc.fr/agvgd\\_input.php](http://agvgd.iarc.fr/agvgd_input.php)), SIFT (<http://blocks.fhrc.org/sift/SIFT.html>), PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) and MutationTaster (<http://www.mutationtaster.org/>). Effects on splicing were investigated in silico using [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html), <http://www.cbs.dtu.dk/services/NetGene2/>, [http://www.genet\\_sickkids.on.ca/~ali/splicesitefinder.html](http://www.genet_sickkids.on.ca/~ali/splicesitefinder.html) and <http://rulai.cshl.edu/tools/ESE/>). All parents available were tested for the mutations found in their affected child by sequence analysis. Non-paternity was excluded in case of *de novo* mutations by multiplex VNTR analysis using the AmpFST® Identifier PCR Amplification kit (Applied Biosystems, Foster City, CA) following the manufacturers protocol.

### RESULTS AND DISCUSSION

In total, we detected 34 (75,6%) heterozygous mutations (25 female, 9 male) in 45 index KS patients. The type of mutations as well as their inheritance are presented in Table 1. In line with the report from Ng et al. (Ng, et al., 2010), the majority are truncating mutations (nonsense or frameshift mutations), although in our study the number of frameshift mutations was relatively high and the number of nonsense mutations low.

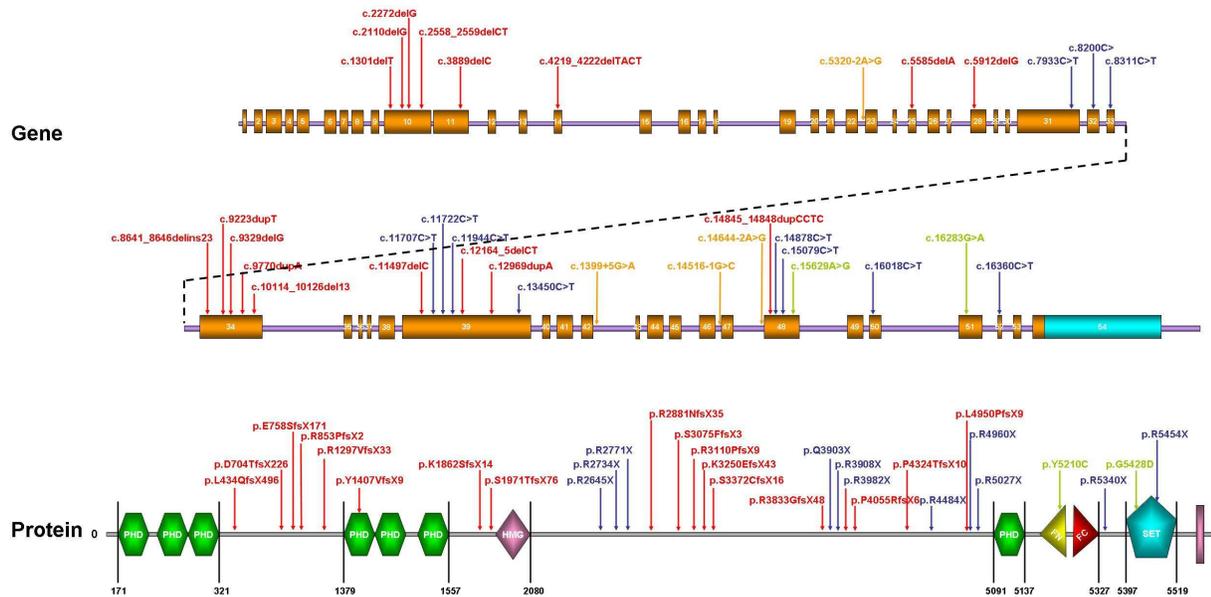
**Table 1.** Frequency of reported *MLL2* mutation types in Kabuki Syndrome

Type of mutation	Frequency (n)	de novo	inherited	no parents available	
Nonsense	this study	11	9	0	2
	Ng et al.	20	7	1	12
Frameshift	this study	17	14	0	3
	Ng et al.	7	3	0	4
Missense	this study	2	2	0	0
	Ng et al.	5	2	0	3
Splice site	this study	4	2	0	2
	Ng et al.	0	0	0	0

In our study we also report the first splice-site mutations (n=4), which adds exon skipping to the spectrum of mechanisms leading to potential abrogation of full-length protein expression. One of the mutations we detected (c.16360C>T (p.Arg5454X), Table 2) was also reported in the original study by Ng et al., all others were novel and private. The 34 mutations detected were randomly distributed across the gene with an expected clustering in some of the larger exons (Table 2, Fig.1). Table 2 presents an overview of the *MLL2* mutations detected, together with the mutations reported by Ng et al. In our cohort two patients carry a missense mutation that each result in amino acid changes at the C-terminal end of the protein in highly conserved protein domains (FYRN and SET

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domains, mutations in green Fig 1.). As both missense mutations were *de novo* in nature and the majority of the *in silico* programs predict these mutations to be pathogenic, it is highly likely that they are causal and affect the function of the protein in a negative way. Additionally, we detected four splice junction mutations affecting the consensus site positions at -1 (patient nr. 26), -2 (patients 7, 27) and +5 (patient 25), respectively (Table 2, mutation in orange Fig 1).



**Figure 1.** Schematic diagram of 34 mutations detected in the *MLL2*-gene in Kabuki syndrome patients. Upper figure: genomic structure of the *MLL2* gene including 54 exons. Coding exons in orange, untranslated region in blue. Mutations in: red; frameshift mutations (n=17), orange; splice-site mutations (n=4), blue; nonsense mutations (n=11), green: missense mutations (n=2). Lower figure: *MLL2* protein structure with conserved protein domains: PHD = plant homeodomain finger, HMG = high mobility group, FN = "FY-rich" domain, N-terminal region, FC = "FY-rich" domain, C-terminal region, SET = SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain, PSET = PostSET domain.

The first three are predicted to result in exon skipping, of exons 47, 48 and 23, the fourth one (c.13999+5G>A) predicts a profound change, but not necessarily skipping of exon 42. This variant was however also *de novo* in nature, and therefore we suspect it to be causal. RNA studies will be performed to determine whether exon skipping is the causal mechanism for all these splice-site mutations. 28 out of 34 mutation carriers represent truncating mutations, either nonsense (n=11) or frameshift (n=17). Although merely three of these mutations localize to one of the proteins functional (conserved) domains, functionally all 28 result in the loss of either the entire C-terminal cluster of functional domains (n=26) or significant parts of it (n=2) (Fig.1). In these cases, truncating mutations abrogate the possibility of complex formation and recruitment and ultimately of methyl transfer onto target lysine residues. As several of the mutations are located within the 50-54 nucleotide perimeter of exon-exon junctions, it is suspected that these will lead to nonsense-mediated decay of mRNA prior to protein synthesis.

Table 3 lists the variants and polymorphisms we detected (n=23) in the coding regions and intron-exon boundaries of the *MLL2* gene in all patients. Five were located in introns, ten were silent variants and eight were missense variants. Twelve of these variants were detected more than once in the KS population and eleven of these were listed as SNP's. Of the remaining eleven, two were intronic, four were silent and five were missense variants (no listing in the SNP database). The latter five variants were all inherited from an asymptomatic parent, four were identified in patients with an already detected pathogenic mutation and the majority of *in silico* programs predicts them to be benign. As such, these variants are unlikely to be causative for KS (Table 3).

Table 2. MLL2 mutations detected in 34/45 KS index patients (**bold**) and as reported by Ng et al.

nr.	Family number	Exon	Mutation	AA change	Mutation type	Inheritance	Reference
1	8312	10	<b>c.1301delT</b>	<b>p.Leu434GlnfsX496</b>	F	de novo	This study
		10	c.1324delC	p.Pro442HisfsX487	F	NA	Ng et al. 2010
2	13070	10	<b>c.2110delG</b>	<b>p.Asp704ThrfsX226</b>	F	de novo	This study
3	8299	10	<b>c.2272delG</b>	<b>p.Glu758SerfsX171</b>	F	de novo	This study
4	12812	10	<b>c.2558_2559delCT</b>	<b>p.Arg853ProfsX2</b>	F	de novo	This study
		11	c.3585_3586insA	p.Pro1196ThrfsX11	F	de novo	Ng et al. 2010
5	15969	11	<b>c.3889delC</b>	<b>p.Arg1297ValfsX33</b>	F	de novo	This study
6	8274	14	<b>c.4219_4222delTACT</b>	<b>p.Tyr1407ValfsX9</b>	F	de novo	This study
		19	c.4843C>T	p.Arg1615X	N	NA	Ng et al. 2010
		19	c.4956_4957insG	p.Glu1654X	N	NA	Ng et al. 2010
7	8433	-	<b>c.5320-2 A&gt;G</b>		S	NA	This study
8	17802	25	<b>c.5585delA</b>	<b>p.Lys1862SerfsX14</b>	F	de novo	This study
		28	c.5875_5891dup17	p.Glu1965GlyfsX88	F	NA	Ng et al. 2010
9	12810	28	<b>c.5912delG</b>	<b>p.Ser1971ThrfsX76</b>	F	de novo	This study
		28	c.6010C>T	p.Gln2004X	N	de novo	Ng et al. 2010
		31	c.6295C>T	p.Arg2099X	N	de novo	Ng et al. 2010
		31	c.6595delT	p.Tyr2199fsX65	F	de novo	Ng et al. 2010
10	17809	31	<b>c.7933C&gt;T</b>	<b>p.Arg2645X</b>	N	de novo	This study
11	13915	32	<b>c.8200C&gt;T</b>	<b>p.Arg2734X</b>	N	de novo	This study
12	17804	33	<b>c.8311C&gt;T</b>	<b>p.Arg2771X</b>	N	de novo	This study
		34	c.8488C>T	p.Arg2830X	N	NA	Ng et al. 2010
13	11103	34	<b>c.8641_8646delins*</b>	<b>p.Arg2881AspfsX35</b>	F	NA	This study
14	17803	34	<b>c.9223dupT</b>	<b>p.Ser3075PhefsX3</b>	F	de novo	This study
15	8358	34	<b>c.9329delG</b>	<b>p.Arg3110ProfsX9</b>	F	NA (M)	This study
		34	c.9961C>T	p.Arg3321X	N	NA	Ng et al. 2010
16	16163	34	<b>c.9770dupA</b>	<b>p.Lys3250GlufsX43</b>	F	de novo	This study
17	8313	34	<b>c.10114_10126del13**</b>	<b>p.Ser3372CysfsX16</b>	F	NA (M)	This study
		38	c.10599_10630del132	p.Val3534GlnfsX11	F	NA	Ng et al. 2010
		38	c.10738C>T	p.Gln3580X	N	NA	Ng et al. 2010
		39	c.11149C>T	p.Gln3717X	N	de novo	Ng et al. 2010
18	17733	39	<b>c.11497delC</b>	<b>p.Arg3833GlyfsX48</b>	F	de novo	This study
19	8346	39	<b>c.11707C&gt;T</b>	<b>p.Gln3903X</b>	N	de novo	This study
20	8355	39	<b>c.11722C&gt;T</b>	<b>p.Glu3908X</b>	N	de novo	This study
		39	c.11794_11797delCAAC	p.Gln3932SerfsX46	F	de novo	Ng et al. 2010
21	13202	39	<b>c.11944C&gt;T</b>	<b>p.Arg3982X</b>	N	de novo	This study
22	8453	39	<b>c.12164_12165delCT</b>	<b>p.Pro4055ArgfsX6</b>	F	de novo	This study
		39	c.12241C>T	p.Gln4081X	N	NA	Ng et al. 2010
		39	c.12697C>T	p.Gln4233X	N	de novo	Ng et al. 2010
		39	c.12703C>T	p.Gln4235X	N	NA	Ng et al. 2010
23	16501	39	<b>c.12969dupA</b>	<b>p.Pro4324ThrfsX10</b>	F	de novo	This study
		39	c.13390C>T	p.Gln4464X	N	de novo	Ng et al. 2010
24	17808	39	<b>c.13450C&gt;T</b>	<b>p.Arg4484X</b>	N	NA	This study
		40	c.13580A>T	p.Lys4527X	N	inherited	Ng et al. 2010
		40	c.13606C>T	p.Arg4536X	N	NA	Ng et al. 2010
25	8438	-	<b>c.13999+5G&gt;A</b>		S	de novo	This study
26	11212	-	<b>c.14516-1G&gt;C</b>		S	NA (F)	This study
27	8393	-	<b>c.14644-2A&gt;G</b>		S	de novo	This study
		48	c.14710C>T	p.Arg4904X	N	NA	Ng et al. 2010
28	8348	48	<b>c.14845_14848dupCCTC</b>	<b>p.Leu4950ProfsX9</b>	F	de novo	This study
29	8347	48	<b>c.14878C&gt;T</b>	<b>p.Arg4960X</b>	N	de novo	This study
30	8817	48	<b>c.15079C&gt;T</b>	<b>p.Arg5027X</b>	N	de novo	This study
		48	c.15195G>A	p.Trp5065X	N	de novo	Ng et al. 2010
		48	c.15217C>T	p.Gln5073X	N	NA	Ng et al. 2010
		48	c.15326G>T	p.Cys5109Phe	M	de novo	Ng et al. 2010
		48	c.15444_15445delTT	p.Phe5149CysfsX9	F	NA	Ng et al. 2010
		48	c.15536G>A	p.Arg5179His	M	de novo	Ng et al. 2010
		48	c.15618T>G	p.Tyr5206X	N	de novo	Ng et al. 2010
31	8423	48	<b>c.15629A&gt;G</b>	<b>p.Tyr5210Cys</b>	M	de novo	This study
		48	c.15641G>A	p.Arg5214His	M	NA	Ng et al. 2010
32	12811	50	<b>c.16018C&gt;T</b>	<b>p.Arg5340X</b>	N	de novo	This study
		50	c.16019G>T	p.Arg5340Lys	M	NA	Ng et al. 2010
33	17805	51	<b>c.16283G&gt;A</b>	<b>p.Gly5428Asp</b>	M	de novo	This study
34	13108	52	c.16360C>T	p.Arg5454X	N	NA	This study, Ng et al. 2010
		52	c.16391C>T	p.Thr5464M	M	NA	Ng et al. 2010
		53	c.16501C>T	p.Arg5501X	N	NA	Ng et al. 2010

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The *MLL2* sequences were compared to the reference sequence of *MLL2* (GenBank accession number NM\_003482.3) with nucleotide numbering starting at the first adenine of the translation initiation codon ATG. NA = not available, (M) mother not a carrier, (F) father not a carrier. \* c.8641\_8646delICGGCACinsGACCTCAATGTCTCTGGACCAGG, \*\* c.10114\_10126delA GCTCACAGCCAG.

**Table 3.** *MLL2* variants/polymorphisms detected in the 45 KS index patients

Exon/ Intron	Variant	AA change	inherited	nr of patients	rs number
int3	c.401-36T>G	-		1	
10	c.2074C>A*	p.Pro692Thr	maternal	1	
10	c.2220G>T	p.Gly740Gly		1	
10	c.2438C>T	p.Pro813Leu		3	rs75226229
11	c.2826C>T	p.Ile942Ile		23	rs2241726
int12	c.4020+13C>G	-		1	
int13	c.4131+83insT	-		21	rs72518091
31	c.6573G>A	p.Thr2191Thr		2	
31	c.7144C>T	p.Pro2382Ser		2	rs3741626
31	c.7378C>T*	p.Arg2460Cys	maternal	1	
31	c.7479G>T	p.Gly2493Gly		29	rs10747559
31	c.7670C>T*	p.Pro2557Leu	paternal	1	
34	c.9726C>T	p.Ala3242Ala		1	
34	c.10192A>G*	p.Met3398Val		1	rs75937132
35	c.10233C>T	p.Asp3411Asp		1	
35	c.10256A>G*	p.Asp3419Gly	paternal	1	
38	c.10671A>G	p.Pro3557Pro		7	rs61942218
39	c.10836G>A	p.Gln3612Gln		23	rs3782357
39	c.12510A>G	p.Pro4170Pro		22	rs3741622
39	c.13071G>C	p.Arg4357Ser	maternal	1	
int39	c.13531-62_65del4	-		4	
41	c.13689C>T	p.Pro4563Pro		5	rs11168830
int52	c.16412+16delG	-		23	rs57079239

\* = detected together with pathogenic mutation

In a preliminary genotype-phenotype correlation study, we compared key clinical features of KS in patients with an *MLL2* mutation to those without an *MLL2* mutation in our cohort (Table 4). The evaluation of facial characteristics was performed by two independent clinical experts (JS, MV) without prior knowledge of *MLL2* mutation carrier status.

**Table 4.** Distribution of Kabuki syndrome clinical features in *MLL2* mutation carriers and non-carriers

Syndrome Feature	<i>MLL2</i> mutation (n = 34)	no <i>MLL2</i> mutation (n = 11)	p-value <sup>1</sup>
Characteristic face <sup>2</sup>	29/34	5/11	<b>0.0143</b>
Growth retardation <sup>3</sup>	24/25	5/10	<b>0.0040</b>
Hypodontia	24/27	4/6	0.2162
Fingertip pads	31/33	7/9	0.1957
Hypotonia	32/33	9/9	(1.0)
Hyperlaxity	32/33	9/9	(1.0)

<sup>1</sup> = two sided Fisher's exact test, significance threshold at P<0.05; <sup>2</sup> defined by elongated palpebral fissures and eversion of the lateral one third of the lower eyelid, an arched or nicked eyebrow with sparseness of hairgrowth in the lateral half, relatively large ears with simplified morphology, a depressed nasal tip and a full lower lip. <sup>3</sup> = threshold -3SD and assessed above 3 years of age; **boldface**. = statistically significant (p<0.05).

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In our cohort KS patients with a *MLL2* mutation were statistically different from patients without a *MLL2* mutation with respect to the presence of the unique facial characteristics and growth retardation (threshold  $-3SD$ ) above the age of 3 years. The other tested characteristics (hypodontia, fingertip pads, hypotonia and hyperlaxity) were equally frequent in both groups. Although larger groups of patients and more elaborate phenotypic characteristics should be evaluated, this study indicates that patients with an *MLL2* mutation may represent a clinical subgroup within the KS patient population.

As pointed out by Ng et al. yet other genes might cause KS and could be identified in patients with KS but without a detectable *MLL2* mutation. Likewise, *MLL2* microdeletions (or microduplications) involving, for example entire exon(s), could still be present in the latter. Although many patients included in this study were assessed previously on various microarray platforms, aberrations at the exon deletion scale could have been missed due to resolution constraints. These patients will be studied by higher resolution microarrays or by semi-quantitative PCR based approaches, focusing on the *MLL2* region.

The ramifications of *MLL2* mutations for early developmental processes, leading to the severe phenotype observed in KS need to be addressed in functional studies following these initial mutation screens. An experimental model such as a mouse *Mll2* gene knock-out would provide valuable information but has yet to be developed. Since there is considerable confusion in the literature it needs to be pointed out here that the existing mouse *Mll2* knock-out is actually the equivalent of a human *MLL4* knock-out due to a recent nomenclature switch that results in mouse *Mll2* now being the ortholog of human *MLL4* (Glaser et al., 2006). The emerging spectrum of *MLL2* mutations to which this study adds 33 novel mutations further provides insight into Kabuki syndrome's etiology. Specific functional studies can now be designed to understand the disease's underlying mechanisms. As stated above, methyl transferase pathways have been implicated before in the pathology of some intellectual disability syndromes. Given the apparent non-redundancy of HMTs within their respective families and their equally non-redundant modes of gene regulation, a specific pathogenicity for each of the pathways involved make HMTs viable targets for further mutational analyses in the very divergent spectrum of, as of yet, unresolved intellectual disability syndromes.

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