

## A mutation screen in patients with Kabuki syndrome

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**Abstract** Kabuki syndrome (KS) is one of the classical, clinically well-known multiple anomalies/mental retardation syndromes, mainly characterized by a very distinctive facial appearance in combination with additional clinical signs such as developmental delay, short stature, persistent fingerpads, and urogenital tract anomalies. In our study, we sequenced all 54 coding exons of the recently identified *MLL2* gene in 34 patients with Kabuki syndrome. We identified 18 distinct mutations in 19 patients, 11 of 12 tested de novo. Mutations were located all over the gene and included three nonsense mutations, two splice-site mutations, six small deletions or insertions, and seven missense mutations. We compared frequencies of clinical symptoms in *MLL2* mutation carriers versus non-carriers. *MLL2* mutation carriers significantly more often presented with short stature and renal anomalies ( $p = 0.026$  and  $0.031$ ,

respectively), and in addition, *MLL2* carriers obviously showed more frequently a typical facial gestalt (17/19) compared with non-carriers (9/15), although this result was not statistically significant ( $p = 0.1$ ). Mutation-negative patients were subsequently tested for mutations in ten functional candidate genes (e.g. *MLL*, *ASC2*, *ASH2L*, and *WDR5*), but no convincing causative mutations could be found. Our results indicate that *MLL2* is the major gene for Kabuki syndrome with a wide spectrum of de novo mutations and strongly suggest further genetic heterogeneity.

### Introduction

Epigenetic control of developmental processes is a fascinating mechanism by which spatial and temporal expression of distinct genes and pathways are regulated (Gibney and Nolan 2010). Alterations of epigenetic mechanisms have been mainly associated with the pathogenesis of cancer, but

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most recently also with the occurrence of congenital malformation syndromes (Fan et al. 2009; Eggermann et al. 2010; Choufani et al. 2010). Recent advances in massive parallel sequencing as well as adopted conceptual strategies, e.g., for analyzing thousands of variants previously identified by whole-exome sequencing have had a great impact on studying Mendelian disorders and have dramatically speeded up gene identification in medical genetics (Ng et al. 2010a; Gilissen et al. 2010; Hoischen et al. 2010; Kalay et al. 2011). An up-to-date example is the identification of de novo dominant mutations in the *MLL2* gene (MIM 602113) using a whole-exome sequencing strategy in patients with Kabuki syndrome (KS) (Ng et al. 2010b).

Kabuki syndrome (MIM 147920) is a rare condition which comprises a typical facial appearance, postnatal short stature, organ malformations, and a varying degree of mental retardation. It was first described independently by two Japanese groups in 1981 (Niikawa et al. 1981; Kuroki et al. 1981). The name of the syndrome refers to the distinctive facial appearance which resembles the make-up of actors in Japanese Kabuki theater. The facial characteristics in Kabuki syndrome include long palpebral fissures with lateral eversion of the lower eyelid, long eyelashes, arched eyebrows with lateral thinning, large, prominent ears with dysplastic helices, and a depressed nasal tip. Additional findings include persistent fetal finger pads, recurrent middle ear infections in infancy, cardiac and renal abnormalities.

*MLL2* is located on chromosome 12q13, and its 54 exons encode a protein of 5,537 amino acids. *MLL2* belongs to the SET1 family of histone methyltransferases involved in epigenetic regulation of development. It acts in a multiprotein complex, and various binding partners within this complex have already been identified (Issaeva et al. 2007). The complex can bind to regulatory sites of genes and induces H3K4 trimethylation, and it is well established that methylation of the histone H3 on lysine K4 is associated with increased transcriptional activation of target genes (Chang et al. 2010). We now confirm that de novo dominant *MLL2* mutations are the major cause of Kabuki syndrome, provide evidence for further genetic heterogeneity, and show that mutations in *MLL2*-binding partners are likely not the cause of *MLL2*-negative Kabuki syndrome.

## Materials and methods

### Patients

34 patients with the clinical diagnosis of Kabuki syndrome, in accordance with the suggested guidelines for clinical diagnosis (Adam and Hudgins 2005), were included in the study. Clinical features of some of the patients have already been published [Utine et al. 2008 (K1816 = patient 1,

K1759 = patient 2, K1764 = patient 5, K1761 = patient 6, K1767 = patient 7, K1770 = patient 8, K1755 = patient 9, K1758 = patient 10, K1765 = patient 11); Armstrong et al. 2005 (K1718)]. All affected individuals or their legal representatives gave written informed consent to the study. The study was performed in accordance with the Declaration of Helsinki protocols. We collected peripheral blood samples from the affected children and parents, after informed consent was obtained, according to the protocols approved by the participating institutions. All the research procedures that followed were in accordance with the ethical standards of the responsible national and institutional committees on human subject research. Written consents for publication of the photographs presented in Fig. 2 were given. DNA from participating family members was extracted from peripheral blood lymphocytes by standard extraction procedures.

### Mutation screening of *MLL2* and relevant candidate genes

The following databases were used to obtain gene information: National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), Ensembl Genome Server (<http://www.ensembl.org/>), UCSC Genome Bioinformatics (<http://www.genome.ucsc.edu/>) and 1000 genomes (<http://browser.1000genomes.org/>). For mutation screening we chose *MLL2*, its paralog *MLL*, and nine additional genes, i.e., *SMAD1*, *CXXC1*, *ASC2*, *ASH2L*, *RBBP5*, *WDR5*, *DPY30*, *PAXIP1*, and *DKK1* that are involved in the ALR complex, *MLL2* regulation and interaction. Primers were designed to amplify exons and adjacent splice sites according to the reference sequences (*MLL2* reference sequence NM\_003482), using ExonPrimer (<http://www.genome.ucsc.edu/>, <http://ihg.helmholtz-muenchen.de/ihg/ExonPrimer.html>). Primer sequences and PCR conditions are available on request. PCR products were sequenced by BigDye Terminator method on an ABI 3730 sequencer. We re-sequenced all identified mutations in independent experiments. The *MLL2* mutation p.S543L in patient K1771 was screened in 100 healthy Turkish control individuals by PCR/restriction digestion using BsrD1. In addition, we tested 103 healthy Turkish control individuals for the presence of the putative *MLL* mutation p.L950F by PCR/restriction digestion using the enzyme MlyI and/or direct sequencing. Paternity testing was performed in 12 father–mother–patient trios using highly informative microsatellite markers D3S642, D3S1038, D3S3611, D10S1269, and D10S1760. Analysis of marker alleles confirmed paternity in all trios analyzed and, thereby, demonstrated a de novo origin of 11 *MLL2* mutations.

In general, we analyzed the non-synonymous variants found in the screen using the servers PolyPhen

(<http://coot.embl.de/PolyPhen/>) and ConSeq (<http://conseq.tau.ac.il/>), and tested for possible splice site alterations ([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.itb.cnr.it/sun/webgene/>, and <http://www.umd.be/HSF/>). The protein sequences were analyzed with the server Pfam (<http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock>) for protein domains. Mutation nomenclature was checked using the Mutalyzer software (<http://www.mutalyzer.nl/2.0/>). Statistical analysis of clinical findings of mutation carriers versus non-carriers was performed using SPSS version 19.0.

## Results

### MLL2 mutation screening

We sequenced all 54 coding exons of the *MLL2* gene by Sanger sequencing in 34 patients who have been clinically diagnosed with Kabuki syndrome according to the suggested clinical diagnostic criteria (Adam and Hudgins 2005). In total, we identified heterozygous *MLL2* mutations in 19 patients (56% overall detection rate), and 18 of these mutations were distinct (Table 1, Supplementary Figure 1). Only the missense mutation c.15461G>A (p.R5154Q) was found as a recurrent de novo mutation in two patients. Mutations included three nonsense mutations (p.G1320X, p.Q2811X, p.R2915X), two splice-site mutations (c.954+1G>T, c.13531–1G>T), six small deletions or insertions leading to a frameshift and premature stop codons (c.1483\_1486delTCTC, c.1512\_1513delTC, c.5268dupG, c.6595delT, c.7289dupT, c.9460delC), and seven missense variants (p.S543L, p.P647Q, p.V1192M, p.H1453R, p.A1718V, p.R5154Q, p.S5498F). Parents were available for testing in 12 families, and we found evidence for de novo origin of the mutations in 11 of these families (Fig. 1). No parental DNA was available for the missense variants p.P647Q, p.A1718V, and p.S543L. None of the three alterations is listed as SNP in databases or the 1000 Genome Project. In addition, p.P647Q and p.A1718V are conserved (ConSeq score: 5 of 9) and predicted as possibly damaging on PolyPhen. As the p.S543L was also present in the mother of patient K1771 (with no obvious clinical phenotype), we tested 100 ethnically matched Turkish controls and were able to show that p.S543L does not represent a common polymorphism. The p.S543L is conserved (ConSeq score: 5 of 9) but not located within a defined domain of *MLL2*, and, therefore, a PolyPhen prediction could not be established. The causative nature of this mutation remains unclear.

Analysis of the distribution of all identified mutations did not show any exon or domain preference within *MLL2* (Fig. 1).

### Additional candidate gene screening

We continued our molecular analysis in 15 *MLL2*-negative patients with Kabuki syndrome. Ten highly relevant candidate genes were chosen based on (1) a direct functional interaction of their gene products with *MLL2* within the multiprotein complex (*SMAD1*, *CXXC1*, *ASC2*, *ASH2L*, *RBBP5*, *WDR5*, *DPY30*, *PAXIP1*), (2) predicted homology and function of the encoded protein (*MLL*), or (3) the description as a target gene (*DKK1*). We directly sequenced 36 exons of *MLL* (NM\_005933, MIM 159555), 7 exons of *SMAD1* (NM\_005900, MIM 601595), 15 exons of *CXXC1* (NM\_001101654, MIM 609150), 15 exons of *ASC2* (NM\_014071, MIM 605299), 16 exons of *ASH2L* (NM\_004674, MIM 604782), 14 exons of *RBBP5* (NM\_005057, MIM 600697), 14 exons of *WDR5* (NM\_017588, MIM 609012), 5 exons of *DPY30* (NM\_032574, MIM 612032), 21 exons of *PAXIP1* (NM\_007349, MIM 608254), and 4 exons of *DKK1* (NM\_012242, MIM 605189).

In the Turkish patient K1772 we found the heterozygous alteration c.2848C>T in exon 3 of *MLL*. This alteration is predicted to substitute a leucine at position 950 by phenylalanine (p.L950F). No further putative mutations were identified in any of the other candidate genes tested. We did not detect the *MLL* c.2848C>T alteration in 103 Turkish control individuals, and found that the variation was inherited from the healthy mother. No second putative mutation could be identified on the father's allele, but we cannot exclude larger deletions and a possible recessive mode of inheritance within this family. No RNA was available from the patient for further analysis of *MLL*. The variation of unclear significance is conserved (ConSeq score: 5 of 9), but is not located within a known protein domain of *MLL* and the PolyPhen program predicted the p.L950F alteration to be "benign" (score 0.9). An accurate prediction concerning its causative nature can hardly be done.

## Discussion

Clinical diagnosis of Kabuki syndrome is mainly based on the combination of unique facial features and the presence of developmental delay. While most patients show mild to moderate mental retardation (Niikawa et al. 1988), normal intelligence as well as severely impaired cognitive function has been reported (Matsumoto and Niikawa 2003; Ho and Eaves 1997). In our study, 95% of patients carrying a mutation in *MLL2* showed some degree of developmental delay, although no formal IQ tests were performed in this study to quantify cognitive capacity. Among our patients with *MLL2* mutation 89% were classified as having a typical facial gestalt in the

**Table 1** Clinical findings in patients with *MLL2* mutation

Patient ID	Freq.	K1715	K1718	K1721	K1722	K1724	K1725	K1747	K1755	K1763	K1766
Sex		f	m	m	f	m	f	f	f	m	m
<i>MLL2</i> mutation		c.3574G>A	c.9460delC	c.16493C>T	c.1512_1513delTC	c.15461G>A	c.13531-1	c.954+1	c.8431C>T	c.4358A>G	c.7289dupT
Clinical findings		p.V1192M <sup>a</sup>	p.L3154X <sup>a</sup>	p.S5498F <sup>a</sup>	p.P506TfsX2 <sup>a</sup>	p.R5154Q <sup>a</sup>	G>T <sup>a</sup>	G>T <sup>a</sup>	p.Q2811X <sup>a</sup>	p.H1453R <sup>a</sup>	p.S2431VfsX3 <sup>a</sup>
Short stature	11/19	+	+	-	+	+	+	+	-	+	-
Microcephaly	11/19	-	-	-	-	+	+	+	-	+	-
Neurodev. symptoms	18/19	+	+	+	+	+	+	+	+	+	+
Developmental delay	18/19	+	+	+	+	+	+	+	+	+	+
Feeding difficulties	13/19	-	+	-	+	+	-	-	+	+	+
Muscular hypotonia	11/17	?	+	+	+	+	?	+	+	+	+
Facial dysmorphism	19/19	+	+	+	+	+	-	+	+	+	+
Typical facial gestalt	17/19	+	+	+	+	+	-	+	+	+	+
Long palpebral fissures	18/19	+	+	+	+	-	+	+	+	+	+
Eversion lat. lower eyelid	15/19	-	+	+	+	-	-	+	+	+	+
Arched eyebrows	17/19	+	+	+	+	+	+	+	+	+	+
Long eye-lashes	17/19	+	+	+	+	+	-	+	+	+	+
Prominent dysplastic ears	18/19	+	+	+	+	+	-	+	+	+	+
Cleft palate	3/19	-	-	-	+	-	-	+	-	-	-
Strabismus	6/19	-	-	+	-	+	-	-	-	-	-
Brain anomalies	7/16	-	-	-	?	n.i.	n.i.	n.i.	-	-	-
Corpus callosum anomalies	2/16	-	-	+	?	n.i.	n.i.	n.i.	-	-	-
Cerebral atrophy	2/16	-	-	-	?	n.i.	n.i.	n.i.	-	+	-
Ventricular dilatation	3/16	-	-	-	+	n.i.	n.i.	n.i.	-	+	-
White matter anomalies	3/16	+	-	-	?	n.i.	n.i.	n.i.	-	-	-
Renal anomalies	7/17	-	-	-	-	n.i.	n.i.	n.i.	-	-	-
Renal malformation	4/17	-	+	+	-	n.i.	n.i.	+	-	-	-
Hydronephrosis	4/17	-	-	+	+	n.i.	n.i.	-	-	+	-
Cong. heart defects	8/18	-	-	-	-	-	n.i.	-	-	+	-
ASD/VSD	5/17	-	n.i.	-	-	-	n.i.	-	-	+	-
PDA/PFO	4/17	-	n.i.	-	-	+	n.i.	-	+	+	-
Others	3/17	-	n.i.	-	-	+	n.i.	-	+	-	-
Other features		-	-	-	-	+	n.i.	-	+	-	-

**Table 1** continued

Patient ID	Freq.	K1715	K1718	K1721	K1722	K1724	K1725	K1747	K1755	K1763	K1766
Fetal finger pads	17/19	+	+	+	+	-	+	+	+	+	+
Frequent otitis	9/19	+	+	+	+	-	-	+	-	+	-
Early puberty	1/15	n.a	+	n.a	-	n.a	n.a	-	-	-	-
Cryptorchidism	4/9	-	-	-	-	+	-	-	-	-	+
Epilepsy	1/19	-	-	-	-	-	-	-	-	-	-
Additional findings		-	DH	-	Scoliosis, CM	Cataract	-	Uvula bifida	-	VUR	HD, BC, P, CD
Patient ID	Freq.	K1767	K1770	K1816	K1818	K1723	K1759	K1761	K1764	K1766	
Sex		f	m	m	m	m	f	f	f	f	
<i>MLL2</i> mutation		c.5268dupG	c.6595delT	c.1483_1486delTCTC	c.15461G>A	c.3958G>T	c.8743C>T	c.5153C>T	c.1940C>A	c.1628C>T	
Clinical findings		p.R1757AfsX31 <sup>a</sup>	p.Y2199IfsX65 <sup>a</sup>	p.S495RfsX434	p.R5154Q	p.G1320X	p.R2915X	p.A1718V	p.P647Q	p.S543L <sup>b</sup>	
Short stature	11/19	-	+	+	-	+	+	+	-	-	
Microcephaly	11/19	+	+	+	+	+	+	+	-	-	
Neurodev. symptoms	18/19	-	+	+	+	+	+	+	+	+	
Developmental delay	18/19	-	+	+	+	+	+	+	+	+	
Feeding difficulties	13/19	-	+	+	-	+	+	-	+	+	
Muscular hypotonia	11/17	-	-	-	+	+	-	-	-	+	
Facial dysmorphism	19/19	+	+	+	+	+	+	+	+	-	
Typical facial gestalt	17/19	+	+	+	+	+	+	+	+	+	
Long palpebral fissures	18/19	+	+	+	+	+	+	+	+	+	
Eversion lat. lower eyelid	15/19	+	-	+	+	+	+	+	+	+	
Arched eyebrows	17/19	+	+	+	+	+	+	+	+	-	
Long eye-lashes	17/19	-	+	+	+	+	+	+	+	+	
Prominent dysplastic ears	18/19	+	+	+	+	+	+	+	+	+	
Cleft palate	3/19	-	-	-	-	-	-	-	-	-	
Strabismus	6/19	-	+	-	-	+	-	+	-	+	
Brain anomalies	7/16	-	-	-	-	-	-	-	-	-	
Corpus callosum anomalies	2/16	-	-	-	-	-	-	-	-	+	
Cerebral atrophy	2/16	-	-	-	-	-	-	+	-	-	
Ventricular dilatation	3/16	-	-	-	-	-	-	+	-	-	
White matter anomalies	3/16	-	-	-	-	-	-	-	+	+	
Renal anomalies	7/17	-	-	-	-	-	-	-	-	-	
Renal malformation	4/17	-	-	+	-	-	-	-	-	-	
Hydronephrosis	4/17	-	-	-	-	-	-	-	-	-	
Cong. heart defects	8/18	-	-	-	-	+	-	-	-	-	

Table 1 continued

Patient ID	Freq.	K1767	K1770	K1816	K1818	K1723	K1759	K1761	K1764	K1771
ASD/VSD	5/17	-	+	-	+	-	+	+	-	-
PDA/PFO	4/17	-	-	-	-	-	-	-	-	+
Others	3/17	-	+	-	-	-	-	-	-	-
Other features										
Fetal finger pads	17/19	+	+	+	+	+	+	+	+	-
Frequent otitis	9/19	-	-	-	+	+	-	-	+	-
Early puberty	1/15	-	-	-	-	n.a	-	-	-	-
Cryptorchidism	4/9	-	+	-	+	-	-	-	-	-
Epilepsy	1/19	+	-	-	-	-	-	-	-	-
Additional findings		CD, JH	DH, HS	CD	AA	-	AA, CD, BD, JH SM, CD, PP PM	AA, CD, BD, JH SM, CD, PP PM	CC, CL	CC, CL

n.a. not applicable, n.i. no imaging, ? unknown, PDA persistent ductus arteriosus, PFO persistent foramen ovale, ASD atrial septal defect, VSD ventricular septal defect, DH diaphragmatic hernia, CM cardiomegaly, VUR vesicoureteral reflux, HD hip dysplasia, BC brachycephaly, P phimosis, CD clinodactyly, JH joint hyperlaxity, HS hypospadias, AA anal atresia, BD brachydactyly, SM stereotypical movements, PP pes planus, PM prematurity, CC colpocephaly, CL cortical lesions

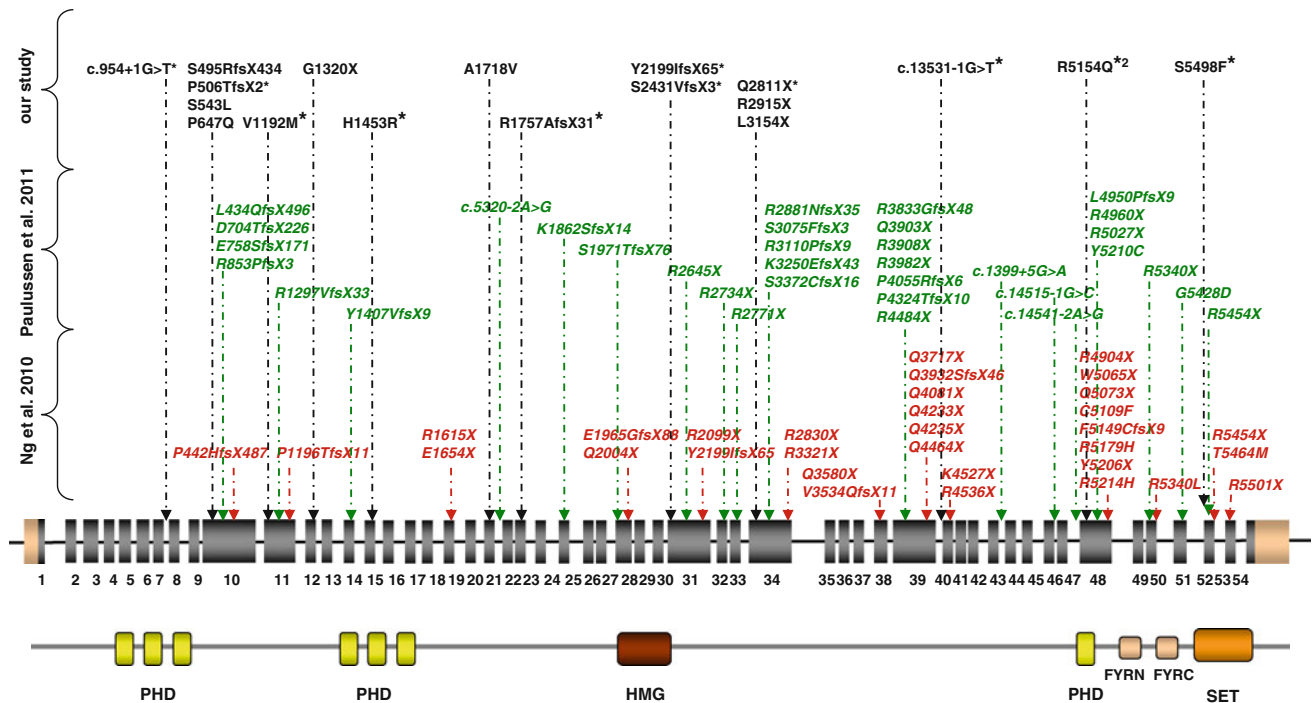
<sup>a</sup> De novo mutation

<sup>b</sup> Inherited from mother

initial physical evaluation (Table 1; Fig. 2), whereas in the patient group without *MLL2* mutation only 60% met these criteria (Table 2). Interestingly, two *MLL2* positive patients did not show the typical facial gestalt. Patient K1725, who carries a de novo splice-site mutation in *MLL2*, only presented with long palpebral fissures and high arched eyebrows, but did not have eversion of lateral lower eyelids, long eye-lashes, and prominent dysplastic ears leading to a not recognizable facial appearance of KS (consent for publication of the photograph was not given). Patient K1771 did not have the typical facial gestalt mainly because of lack of high arched eyebrows (again, consent for publication of the photograph was not given). In addition, it is important to mention that not all *MLL2*-positive patients with KS presented with long palpebral fissures, a hallmark of the facial phenotype (Table 1, Adam and Hudgins 2005). These findings clearly indicate a certain clinical variability regarding facial features in Kabuki patients and make clinical evaluation more difficult. The overall mutation detection rate in our study was 56%. Consideration only of patients with typical facial gestalt in addition to other clinical features would have raised the detection rate to approximately 70% (19 of 27) (Tables 1, 2), but in that case at least one individual would have been missed. Quantitative testing of *MLL2* for larger deletions or duplications, e.g., by MLPA, might increase the mutation detection rate. In a recent *MLL2* mutation screen the detection rate was comparable with approximately 75% (Paulussen et al. 2011).

In contrast to both the previous studies, we did find a higher percentage of putative causative missense mutations in *MLL2* (7/19 = 37%) compared to the studies by Paulussen et al. (2/34 = 6%) and Ng et al. (5/32 = 16%). Taking into consideration that the causative nature of identified missense mutations in our study and the study by Ng et al. could not be finally proven by de novo occurrence or inheritance from an affected parent for all missense mutations, definite frequencies of missense mutations could be much lower in both studies (3/19 = 16% and 3/32 = 9%, respectively). In prior studies, only truncating mutations were detected in the N-terminal region of *MLL2* and missense mutations were located only within functionally important domains of the C-terminal part of *MLL2*. Interestingly, two de novo missense mutations, p.V1192M and p.H1453R, we detected in our study are located more in the N-terminus of the *MLL2* protein not affecting a conserved functional protein domain of *MLL2* suggesting a different underlying functional effect of these mutations. It will be important in future studies to elucidate the effect of these mutations on protein structure and function.





**Fig. 1** Distribution of *MLL2* mutations. Schematic view of the *MLL2* protein domains, all coding exons, and localization of identified *MLL2* mutations. The mutations detected in the present study are shown on top and de novo mutations are marked with an asterisk.

One patient, K1771, who carried the maternally transmitted p.S543L missense variant, showed most but not all of the key features of KS, while his mother was phenotypically unremarkable. Although the causative nature of this mutation remains uncertain, it might point toward incomplete penetrance of dominantly inherited *MLL2* mutations, a finding that has already been described in other autosomal dominant developmental syndromes (Dodé and Hardelin 2010, Francis and Antzlevitch 2005).

Postnatal short stature is a common finding in KS, which we observed in 58% of patients with *MLL2* mutations. Microcephaly has been reported to be present in 25–30% of patients with KS (Matsumoto and Niikawa 2003). We discovered the prevalence to be as high as 58% among our patients with *MLL2* mutations, which suggests that this feature might have been underestimated in the past. Muscular hypotonia and feeding problems are frequent in KS and were seen in 65 and 68% of our patients. 44% of our patients in the *MLL2* mutation-positive group who did undergo brain imaging showed brain abnormalities, namely white matter anomalies (20%), ventricular dilatation (19%), cerebral atrophy (13%) and corpus callosum a-/dysgenesis (13%). Brain anomalies had a higher prevalence in the *MLL2* mutation-negative group (60%), as did epi-

lepsy (47 vs. 5%), suggesting the presence of alternative entities in this group. Renal anomalies have been reported to occur in over 25% of patients with KS (Adam and Hudgins 2005). In this study, renal anomalies were present in 41% of our patients with *MLL2* mutations versus 7% in the mutation-negative group, signifying that renal malformations are frequent in KS and should be looked for in KS patients who have not had a renal sonogram. The incidence of congenital heart defects in KS has been estimated at 40–50% (Adam and Hudgins 2005). We confirm this frequency, having found congenital heart defects in 47% of our *MLL2*-positive patients.

Other frequent findings in KS patients include persistent fingerpads, an indicative diagnostic criterion which we found in 90% of patients with *MLL2* mutations, and a susceptibility to middle ear infections, which 47% of our patients in the *MLL2* mutation-positive group suffered from. In the future, it will be interesting to specify epigenetic alterations and altered expression of genes responsible for the various symptoms seen in patients with KS.

We performed statistical analysis of clinical symptoms observed in *MLL2* mutation carriers versus non-carriers. Although the number of patients in our cohort was limited, short stature and renal anomalies were more frequently present in *MLL2* mutation carriers ( $p = 0.026$  and  $0.031$ ,



**Fig. 2** Faces of *MLL2* mutation-positive patients with Kabuki syndrome. Facial appearance of 12 patients with Kabuki syndrome harboring heterozygous *MLL2* mutations. Typical characteristics

include long palpebral fissures with lateral eversion of the lower eyelid, long eyelashes, arched eyebrows with lateral thinning, depressed nasal tip, and large, prominent ears with dysplastic helices

respectively; Fisher's exact test) (Table 3). In addition, *MLL2* mutation carriers obviously presented more often with a typical facial gestalt (17/19) compared with non-carriers (9/15). However, this finding was not statistically significant ( $p = 0.1$ ; Fisher's exact test). Our data are in agreement with the study by Paulussen et al. describing statistically significant differences between *MLL2* mutation carriers and non-carriers in the presence of growth retardation and distinct facial appearance.

Our candidate gene screen did not provide convincing evidence for a second Kabuki gene, although our data

show that further genetic heterogeneity is likely. We identified a single alteration in *MLL* with a yet unclear causative role, but this variation is predicted to be benign and was inherited from the unaffected mother. Although unlikely, we cannot completely rule out a recessive inheritance and a second mutation inherited from the father which was not identified by conventional Sanger sequencing of *MLL* coding exons. Use of whole exome sequencing in *MLL2*-negative patients will likely prove genetic heterogeneity in Kabuki syndrome in the near future.



**Table 2** Clinical findings in patients without *MLL2* mutation

Patient ID	Freq.	K1760	K1772	K1768	K1757	K1762	K1820	K1815	K1756	K1758	K1765	K1720	K1769	K1819	K1824	K1833
Sex		f	m	m	f	f	m	f	f	f	m	m	m	f	m	f
<i>MLL2</i> mutation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical findings																
Short stature	3/15	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-
Microcephaly	8/16	-	-	+	+	+	+	+	-	-	-	+	+	-	-	-
Neurodev. symptoms	14/15															
Developmental delay	14/15	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Feeding difficulties	9/15	+	+	-	+	-	-	+	+	-	+	+	-	-	+	+
Muscular hypotonia	6/15	+	+	-	+	+	-	-	-	-	-	+	-	-	-	+
Facial dysmorphism	15/15															
Typical facial gestalt	9/15	+	-	-	+	-	+	+	+	+	-	+	-	-	-	+
Long palpebral fissures	15/15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eversion lat. lower eyelid	13/15	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Arched eyebrows	14/15	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Long eye-lashes	13/15	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Prominent dysplastic ears	15/15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cleft palate	1/15	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Strabismus	6/15	+	-	-	-	-	+	-	+	+	-	-	-	+	+	+
Brain anomalies	6/10															
Corpus callosum anomalies	2/10	-	-	n.i.	-	-	n.i.	-	-	+	-	n.i.	+	-	n.i.	n.i.
Cerebral atrophy	3/10	+	+	n.i.	+	-	n.i.	-	-	-	-	n.i.	-	-	n.i.	n.i.
Ventricular dilatation	2/10	-	-	n.i.	+	-	n.i.	-	+	-	-	n.i.	-	-	n.i.	n.i.
White matter anomalies	3/10	-	+	n.i.	+	-	n.i.	-	+	-	-	n.i.	-	-	n.i.	n.i.
Renal anomalies	1/14															
Renal malformation	0/14	-	-	-	-	-	-	-	-	-	-	n.i.	-	-	-	-
Hydronephrosis	1/14	-	-	-	-	-	-	-	-	-	-	n.i.	-	-	-	+
Cong. heart defects	4/15															
ASD/VSD	4/15	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+
PDA/PFO	2/15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Others	1/15	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Other features																
Fetal finger pads	10/15	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-
Frequent otitis	4/15	-	-	-	-	-	+	+	+	-	-	+	-	+	+	-
Early puberty	0/15	-	-	-	-	-	-	-	-	-	-	n.a	-	n.a	n.a	n.a
Cryptorchidism	2/7	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
Epilepsy	7/15	-	-	-	+	-	+	-	+	+	+	-	+	+	+	-
Additional findings		CA, WN	PP, MC	ADHS	HD	JH	JH, SD	-	HL	PP	-	DH	SM	DBA	ITP	HXD

n.a. not applicable, n.i. no imaging, ASD arial septal defect, VSD ventricular septal defect, PDA persistent ductus arteriosus, PFO persistent foramen ovale, CA cerebral angiotoma, WN webbed neck, PP pes planus, MC macrocephaly, ADHS attention deficit hyperactivity syndrome, HD hip dysplasia, JH joint hyperlaxity, SD Sprengel deformity, HL hearing loss, DH diaphragmatic hernia, SM stereotypical movements, DBA delayed bone age, ITP immune thrombocytopenia, HXD hexadactyly

**Table 3** Phenotypic differences between *MLL2* mutation carriers and non-carriers

<i>MLL2</i> mutation	Positive	Negative	
Clinical findings	Frequency (%)	Frequency (%)	<i>p</i>
Short stature	58	20	0.038
Microcephaly	58	53	>0.05
Neurodevelopmental symptoms	95	93	>0.05
Facial dysmorphism	100	100	>0.05
Typical facial gestalt	89	60	>0.05
Brain anomalies	44	60	>0.05
Renal anomalies	41	7	0.045
Congenital heart defects	47	27	>0.05
Fetal finger pads	90	66	>0.05

Taken together, our results confirm that *MLL2* is the major gene for Kabuki syndrome with a wide spectrum of de novo mutations.

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