Original article

Molecular karyotyping in 17 patients and mutation screening in 41 patients with Kabuki syndrome

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Running Title: Molecular karyotyping in Kabuki syndrome

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Abstract

Kabuki syndrome (KS, OMIM 147920) also known as Niikawa-Kuroki syndrome, is a multiple congenital anomaly/mental retardation syndrome characterized by a distinct facial appearance. The cause of KS has been unidentified even by whole genome scan with array comparative genomic hybridization (CGH). In recent years, high resolution oligonucleotide array technologies enable us to detect fine copy number alterations. In 17 patients with KS, molecular karyotyping was performed with GeneChip 250K NspI array (Affymetrix) and CNAG (Copy Number Analyser for GeneChip). It revealed seven copy number alterations, three deleted region and four duplicated region, among the patients with the exception of registered copy number variants (CNVs). Among the seven loci, only the region of 9q21.11-q21.12 (~1.27 Mb) involved coding genes, i.e., *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*. Mutation screening for the genes detected 10 base substitutions consisting of seven single nucleotide polymorphisms (SNPs) and three silent mutations in 41 patients with KS. Our study could not reveal causative genes for KS, but the locus of 9q21.11-q21.12 in association with cleft palate may contribute to its manifestation of KS in the patient. Since various platforms on oligonucleotide arrays have been developed, higher resolution platforms will need to be applied to search tiny genomic rearrangements in patients with KS.

Key words: Kabuki syndrome/Microdeletion/Molecular karyotyping/Mutation screening/Niikawa-Kuroki syndrome

Introduction

Kabuki syndrome (KS, OMIM 147920) also known as Niikawa-Kuroki syndrome, is a multiple congenital anomaly/mental retardation (MCA/MR) syndrome characterized by a distinct facial appearance, skeletal abnormalities, joint hypermobility; dermatoglyphic abnormalities, postnatal growth retardation, recurrent otitis media and occasional visceral anomalies.[1,2] The prevalence was estimated to be 1/32,000 in Japan[3] and 1/86,000 in Australia and New Zealand.[4] Although most cases were sporadic, at least 14 familial cases have been reported. It is thought that KS is an autosomal dominant disorder considering the equal male-to-female ratio of patients and parent-child transmission pattern in some familial cases.[5]

The cause of KS remains unknown, even though at least 400 patients have been known in a variety of ethnic groups since 1981.[3-7] Some works have ruled out several loci, e.g., 1q32-q41, 8p22-p23.1 and 22q11, from the candidate for KS.[8-13] A study of array-based comparative genomic hybridization (CGH) showed a disruption of *C20orf133(MACROD2)* gene by ~250 kb deletion on a patient with KS[14], but the following mutation screening for the gene failed to find pathogenic base change within exons in other 19 patients with KS[14] and in 43 Japanese patients.[15] Another study of array CGH with 0.5 – 1.2 Mb resolution reported that 2q37 deletions were detected in two patients with Kabuki-like features but their facial feature were not typical for KS.[16] To date, no concordant specific lesion have been found by whole genome scan with array CGH of bacterial artificial chromosome (BAC) clone with 0.5 - 1.5 Mb resolution.[16-18]

Chromosomal aberration analysis by high resolution oligonucleotide array technologies in recent years, called molecular karyotyping, enable us to detect submicroscopic pathogenic copy number alterations which were undetectable by even BAC

array CGH.[19,20] Since not a few MCA/MR syndromes are due to chromosomal copy number aberration, we hypothesize that some sort of microdeletion/microduplication cause KS. Herein we report the results of molecular karyotyping in 17 patients with GeneChip 250K array and mutation screening of candidate genes in 41 patients with KS in Japan.

Materials and methods

Subjects

The subjects for molecular karyotyping consisted of 18 patients (nine girls and nine boys) at entry. The subjects for mutation screening consisted of 41 patients (20 girls and 21 boys) including aforementioned 18 patients. The diagnoses of KS were confirmed by experts of clinical genetics, though written permission for the use of facial photographs in publications was not obtained. These Japanese patients showed normal karyotype at 400 band level, who were previously reported with no pathogenic genome copy number change by 1.5 Mb-resolution BAC array CGH.[18] Genomic DNA was isolated by the standard method from their peripheral blood leukocytes or in part from their lymphoblastoid cell lines. Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

Molecular karyotyping

DNA oligomicroarray hybridization using the GeneChip® Human Mapping 250K Nsp Array (Affymetrix, Santa Clara, CA, USA) was performed for 18 patients with KS following the provided protocol (Affymetrix). Data were analyzed to detect copy number aberration using GTYPE (GeneChip Genotyping Analysis Software) and visualized with

CNAG (Copy Number Analyser for GeneChip) ver.3.[21] References for non-paired analysis of CNAG were chosen from eight unrelated individuals of HapMap samples from Affymetrix website (http://www.affymetrix.com/support/). The resolution of this procedure was estimated as ~30 – 100 kb. CNAG ver.3 was linked with the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) assembly May 2004, and then its physical position was referred to the data assembly on March 2006 in the UCSC genome browser after adjustment.

Validation of deletion

Quantitative polymerase chain reaction (qPCR) analysis to validate deletions was run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) using an intercalating dye, SYTO9 (Molecular probes, OR, USA), which is an alternative to SYBR green I.[22] Absolute quantification with second derivative max method was used for quantification. A standard curve of amplification efficiency for each set of primers was generated with a serial dilution of genomic DNA. A corrected gene dosage was given as a ratio of a target gene divided by an internal control gene. The copy number was obtained from a calibration under the assumption that the control genome was diploid.

Target genes of copy number aberration were as follows: *SUMF1* (for patient K9); *MAMDC2* (for patient K16); *CETN1* (for patient K34). The primer sequences of these genes are available in online supplementary file. Internal control diploid genes were *OAZ2* and *USP21*. Primer sets of the control genes for genomic DNA were selected from Real Time PCR Primer Sets website (http://www.realtimeprimers.org/). The control genes were confirmed to have no copy number variants on Database of Genomic Variants (DGV) updated

on June 26, 2008 (http://projects.tcag.ca/variation/). BLAST searches confirmed all primer sequences specific for the gene.

Samples were analyzed in triplicate in 384 well format in a 10 μl final volume containing about 2 ng of genomic DNA, 0.5 μM forward primer, 0.5 μM reverse primer, 0.1 Units TaKaRa ExTaq HS version (TaKaRa, Kyoto, Japan), 1x PCR buffer, 200 μM dNTP, and 0.5 μM SYTO9. The amplification conditions consisted of first denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec, and extension at 72°C for 15 sec. Data was analyzed with using LightCycler 480 Basic Software (Roche Diagnostics) and melting curve was checked to eliminate nonspecific products from the reaction.

Mutation screening of candidate genes

Candidate genes, identified within a detected deletion, consisted of four genes: *TRPM3* (NM_001007471 and NM_206946), *KLF9* (NM_001206), *SMC5* (NM_015110), and *MAMDC2* (NM_153267) located at 9q21.12-q21.11. The entire coding region and splice junctions of the genes were sequenced on an automated sequencer 3130xl (Applied Biosystems, Foster City, CA, USA) using BigDye ver.3.1 (Applied Biosystems). Genomic sequences were retrieved from the UCSC genome browser (assembly: Mar, 2006). PCR primers were designed with the assistance of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The primer sequences are available in online supplementary file. Resultant electropherograms were aligned using ATGC v3.0 (Software Development, Tokyo, Japan) and inspected visually to find DNA alterations.

In silico analysis

Relations among deleted genes were assessed with online software PANTHER (Protein Analysis Through Evolutionary Relationships, http://www.pantherdb.org) to determine whether the genes involve some developmental pathway or biological process.[23] Novel synonymous base substitutions found in the mutation screening were examined for their potential activation of cryptic splice site by comparison between wild type allele and mutated allele using the GeneSplicer program (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml).

Results

Molecular karyotyping and Validation of deletion

The entries of molecular karyotyping were 18 patients with KS (K1, K3, K5, K6, K7, K8, K9, K11, K12, K13, K16, K18, K20, K21, K22, K23, K34, and K38). We eliminated the data of patient K3 from copy number analysis, because it showed low quality data, i.e. single nucleotide polymorphism (SNP) call rate 82.51% and quality control performance detection rate 74.09%, probably due to DNA degradation during the long term storage. The other patients showed high call rates enough for copy number analysis (SNP call rate, 90.07%-97.72% and detection rate, 91.52%-99.77%). We identified nine deleted regions, which lengths were between ~35 kb and ~1.27 Mb, and nine duplicated regions, its length between ~72 and ~495 kb, in the 17 patients analyzed (Table 1). As for detected nine duplications, five of them were concordant to several observed gains in DGV and four of them in each patient did not contain any known genes.

Interestingly, the deleted region of 9q21.11-q21.12 (~1.27 Mb in patient K16), which had not been registered in DGV, harbored four known genes: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2* (Figure 1d). The deletion of 3p26.2 (~205 kb in patient K9, Figure 1a) had involved in a noncoding exon of *SUMF1*gene. The deletion of 18p11.32 (~35 kb in patient K34, Figure 1b) containing *CETN1* gene had one registration in DGV as Variation_5044, which described only one observed loss and 14 observed gain of 95 individuals. The deletion of 4q13.2 (~1.26 Mb in patient K23, Figure 1c) and 20p12.1 (~152 kb in patient K6) did not carry any coding exon of any gene. The regions of 14q11.2 (~116 kb in patient K5) and 15q11.2 (~972 kb in patient K1 and K23) were non-pathologic deletions with the many registrations as observed losses in DGV.

To validate the deletion of the detected region, we confirmed its loss of heterozygosities of SNP probes there with GTYPE (data not shown) and performed qPCR. The regions of *SUMF1* on 3p26.2 (for patient K9) and *MAMDC2* on 9q21.11-q21.12 (for patient K16) had one copy in each patient compared with unaffected individuals (Figure 2). The deletion of *CETN1* on 18p11.32 (for patient K34) was inherited from his unaffected mother. Because the parents' samples of the patient K16 were unavailable, it was unable to examine whether the deletion of 9q21 was *de novo* or not. But the deletion was not found in 95 normal Japanese individuals by qPCR (data not shown).

In consequence of this copy number analysis, we considered the next four genes as candidate genes for KS: *TRPM3* (Transient receptor potential cation channel, subfamily M, member 3), *KLF9* (Kruppel-like factor 9), *SMC5* (Structural maintenance of chromosomes protein 5) and *MAMDC2* (MAM domain containing 2).

Mutation screening and *in silico* analysis

Table 2 shows the results from mutation screening of the four candidate genes in 41 patients with KS. Ten base substitutions were found in the 41 patients, consisting of six registered SNPs, one unregistered SNPs and three silent mutations. In addition, *SUMF1* (NM_182760) and *CETN1* (NM_004066) were also screened but no mutations were detected (data not shown).

We checked the three silent mutations for splice site alteration using GeneSplicer program, but no activation of cryptic splice site was predicted. While PANTHER classification of the four candidate genes did not show significant correlation for biological processes or pathway because of its small scale in number, some genes associated with developmental biology, i.e. DNA repair (*SMC5*) and mRNA transcription regulation (*KLF9*).

Discussion

We used high resolution oligonucleotide array of GeneChip 250K NspI with the resolution of 30 - 100 kb setting and tried to find causative deletions or mutated genes for KS. Our molecular analysis did not show the causative gene for KS with strong evidence, but we identified a locus possibly contributed to KS.

The deletion in patient K16, its length ~1.27 Mb in size at 9q21.11 - q21.12, harbored four known gene: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2* (Figure 1d). Unfortunately her parents' DNAs were unavailable, but the region is unlikely copy number variant (CNV) because it has not been known as CNV in DGV and additionally the deletion was not found in 95 normal Japanese individuals by qPCR.

Since mutation screening in 41 patients with KS revealed no pathogenic base

substitution in these genes, we cannot state that these genes are major genetic factors for KS. However, it is presumable that the genes have some etiological roles for KS because of its genetic heterogeneity. Ontology of PANTHER classification suggested the three genes were associated with developmental biology, such as mRNA transcription regulation. Moreover, the 1.27 Mb region of 9q21 was included in previously reported candidate locus of cleft lip/palate by meta-analysis of linkage analysis.[24] The patient K16 actually had velopharyngeal insufficiency due to the submucous cleft palate. Therefore, it is reasonable to consider the deleted genes cooperated with development of a cleft palate, which is often accompanied by KS.

Although the ~152 kb deletion within intron 5 of *C20orf133 (MACROD2)* in patient K6 did not involve any coding exon and her parents' DNAs were unavailable, the deletion was neither registered as CNV in DGV nor found in 95 normal Japanese individuals by qPCR (data not shown). Maas et al[14] reported *de novo* ~250 kb deletion including exon 5 of *C20orf133 (MACROD2)* in a patient with KS. Direct sequencing for the gene in other 62 patients with KS did not detect mutations[14,15], but the gene may be one of the causative genes for KS in consideration of its genetic heterogeneity.

We focused this study for KS on deletion/duplication detected by oligonucleotide array and mutation screening of the coding genes within the region. One limitation of the study is its resolution. As a matter of course, higher resolution array can detect smaller genomic rearrangements which were undetectable in a same patient, as we showed here compared with previous study of BAC array CGH.[18] While SNP probes are useful to examine loss of heterozygosity as a collateral evidence in deletions, unevenly-distributed probes of the SNP array have disadvantage for CNV detection. Since various platforms on oligonucleotide

array have developed, higher resolution platforms will need to be applied to search tiny genomic rearrangements in patients with KS. Another limitation is that we assumed a single copy number change caused KS. It remains to be elucidated that whether CNV association[25] contribute to manifestations of KS. If the further investigation with refined array technologies could not find the etiology of KS, the direction of study for KS will have to be changed to find *de novo* sequence alteration or methylation aberration including even in the noncoding genomic regions.

In summary, we applied molecular karyotyping with GeneChip 250K array to detect copy number aberrations in 17 patients with KS and screened the four candidate genes in 41 patients with KS. We could not identify causative DNA alteration for KS, but the locus, 9q21.11-q21.12 including *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*, may contribute to the cleft palate of KS. Further investigations will be needed since various array platforms have the potential to specify genomic alterations for KS.

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Titles and legends to figures

Figure 1 Chromosome view of CNAG analysis.

Each dots represent fluorescent intensity on each SNP probe of GeneChip 250K NspI array (Affymetrix). Solid lines indicate copy number analyzed with CNAG. Arrows show detected deletions.

(a) Chromosome 3 of patient K9, ~205 kb deletion in 3p26.2 involving an exon of *SUMF1*gene.

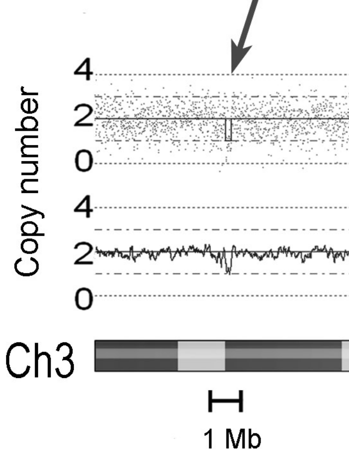
(b) Chromosome 18 of patient K34, ~35 kb deletion in 18p11.32, containing *CETN1* gene.

(c) Chromosome 4 of patient K23, ~1.26 Mb deletion in 4q13.2, not involving any known gene.

(d) Chromosome 9 of patient K16, ~1.27 Mb deletion in 9q21.11-q21.12, harboring four genes: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*. The UCSC genome browser denotes the cytobands, genes, and probe setting of Affymetrix 250K NspI array within the region. No copy number variation was registered here in Database of Genomic Variants updated June 26, 2008.

Figure 2 Validation of deletion with quantitative PCR.

Quantitative PCR confirmed a loss of one copy in each patient: *SUMF1* at 3p26.2 for patient K9; *MAMDC2* at 9q21.1 for patient K16; *CETN1* at 18p11.32 for patient K34. The deletion of patient K34 was inherited from his unaffected mother. UA, unaffected individual. Error bars, s.d.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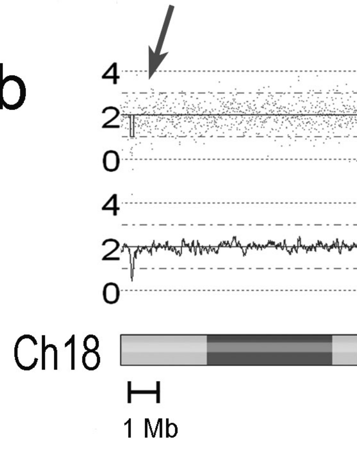
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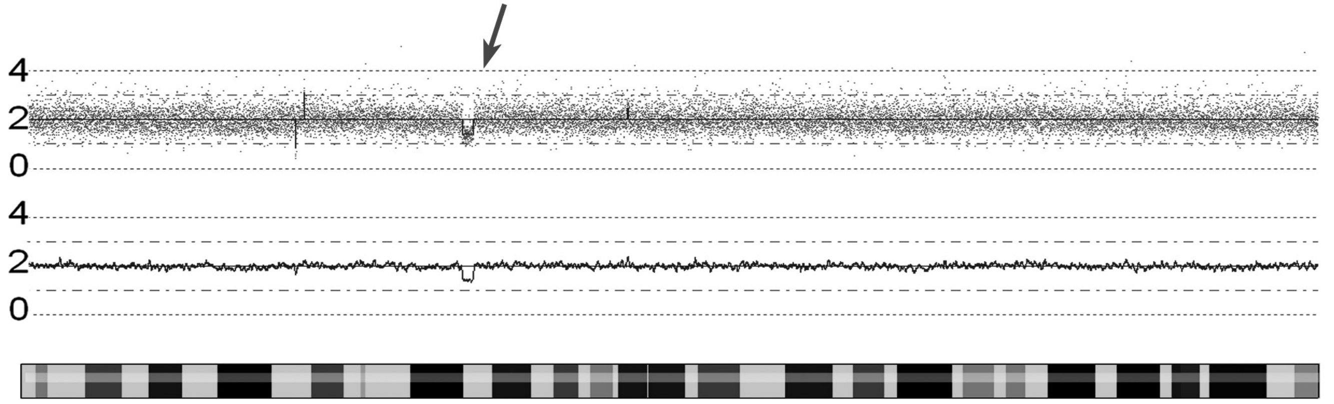
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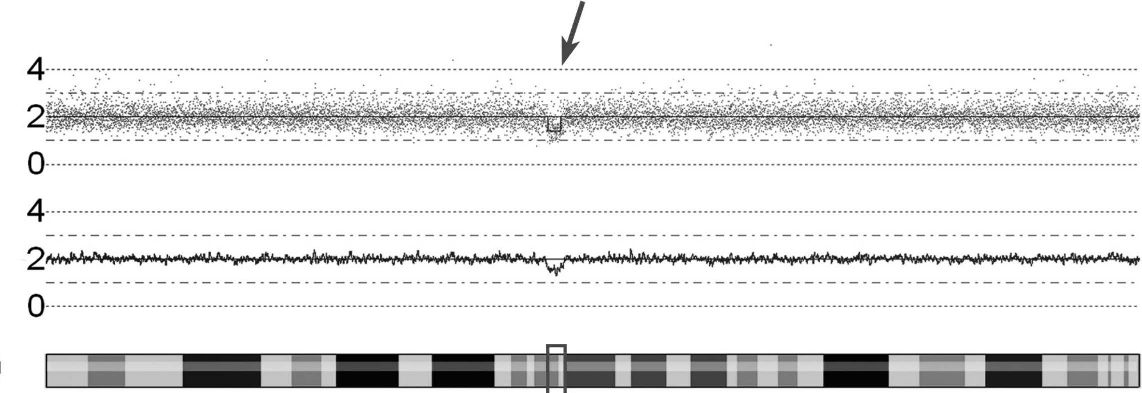
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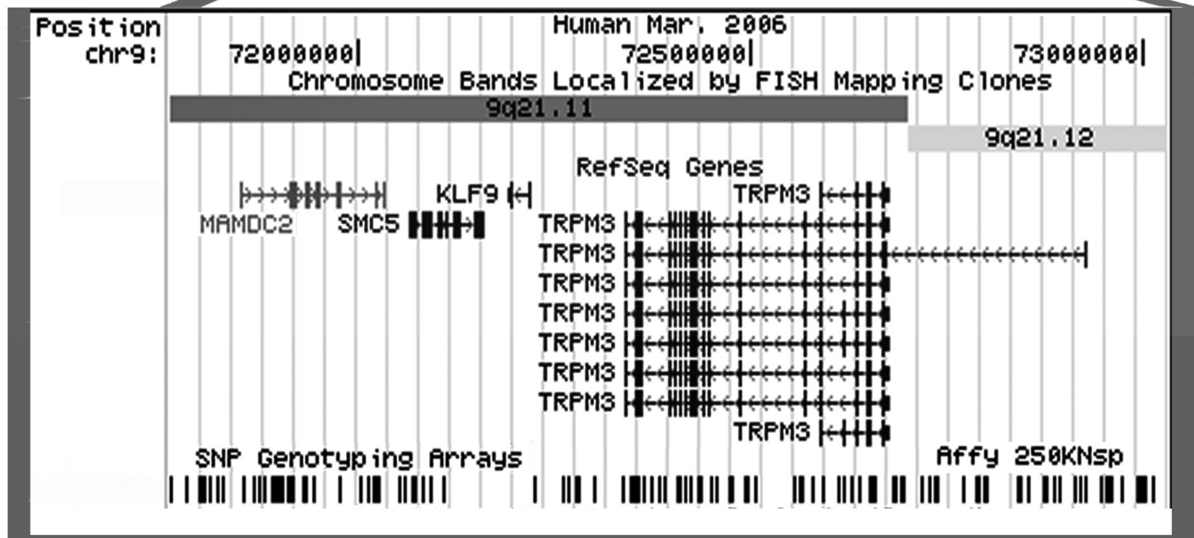
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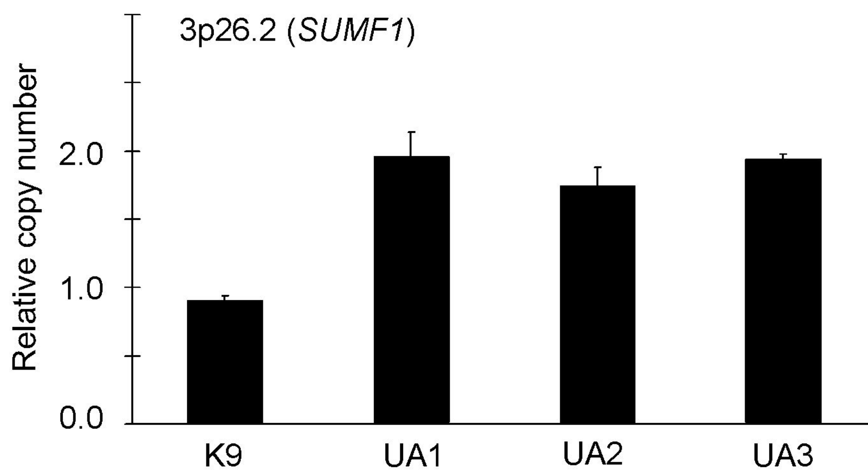
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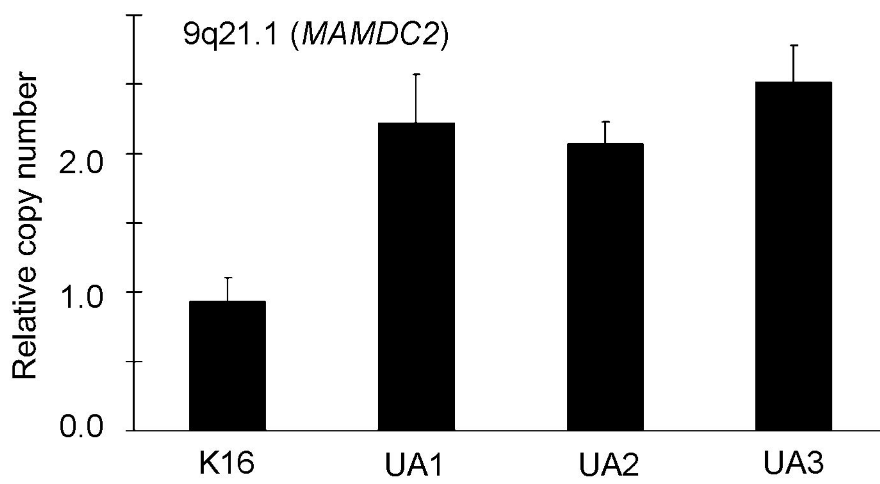
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Relative copy number
0.0
1.0
2.0
K16
UA1
UA2
UA3

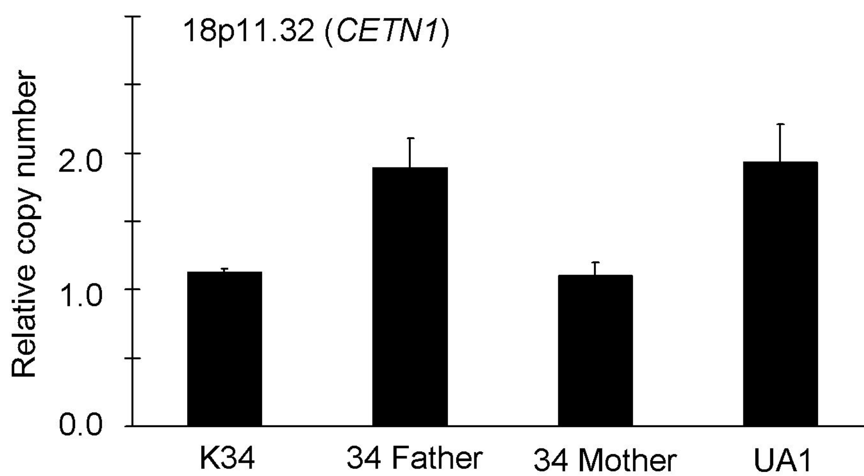
18p11.32 (CETN1)
Relative copy number
0.0
1.0
2.0
K34
34 Father
34 Mother
UA1

Table 1. Detected genomic copy number aberrations in 17 patients with Kabuki syndrome.

Cytoband	Patient(s) ID	CN State	Length	Physical position		Involving Gene(s)	Concordant Loss/Gain on DGV
				Start	End		
3p26.3	K7	1	460 kb	1435279	1895554	N.R.	Variation_8235
3p26.2	K9	1[a]	205 kb	4009368	4214847	*SUMF1*	Variation_8973, 8975, 30169
4q13.2	K23	1[a]	1.26 Mb	66329014	67591611	N.R.	N.R.
5q21.2-q21.3	K22	1	281 kb	104301325	104581898	N.R.	Variation_3568
9q21.11-q21.12	K16	1[a]	1.27 Mb	71760296	73031176	*TRPM3, KLF9, SMC5, MAMDC2*	N.R.
14q11.2	K5	1	166 kb	19336854	19502641	*OR4N2, OR4K2, OR4K5, OR4K1*	Variation_0376, 7028, 8094, 9234, 9235
15q11.2	K1, K23	1	972 kb	19356830	20329239	*OR4M2, OR4N4, LOC65D137*	Variation_0318, 3070, 8265, 9251, 9254, 9256
18p11.32	K34	1[a]	35 kb	545074	580003	*CETN1*	Variation_5044
20p12.1	K6	1[a]	152 kb	14993412	15145890	*C20orf133 (MACROD2)* [b]	N.R.
4q12	K5	3	104 kb	54251599	54355281	N.R.	N.R.
8q11.21	K7	3	171 kb	50641101	50812548	N.R.	Variation_2751, 3731, 8601, 37765
10p15.2-p15.1	K5	3	142 kb	3663600	3805292	N.R.	N.R.
13q31.1	K6	3	72 kb	82451568	82523728	N.R.	N.R.
15q11.2	K7, K9, K12	3	877 kb	19112164	19989036	*CXADRP2, POTEB*	Variation_3070, 3951, 8784, 30670, etc.
15q25.1	K9	3	165 kb	76992181	77156751	*CTSH, RASGRF1*	Variation_3970, 7073
16q21	K13	3	283 kb	58508008	58791285	N.R.	N.R.
17q12	K7	3	495 kb	31428390	31923810	*CCL3, CCL4, CCL3L1, CCL3L3, CCL4L1, CCL4L2, TBC1D3B, TBC1D3C, TBC1D3G*	Variation_3142, 4031, 8841, 30824, etc.
22q11.22	K5, K12	3	278 kb	20907806	21186081	*VPREB1, ZNF280B*	Variation_5356, 34540

CN, Copy number; DGV, Database of Genomic Variants; N.R., no registeration in UCSC genes or DGV;

[a] Validated by quantitative PCR.

[b] Deleted region was within intron 5 of the *C20orf133 (MACROD2)* and did not involve any coding exon.[15]

Table 2 Mutation screening of candidate genes in 41 patients with Kabuki syndrome

Involving gene(s)	Base substitution	Amino acid change	Patient(s) with KS		dbSNP	Allele frequency among unaffected Japanese[a]	Results of mutation screening
			Homo	Hetero			
TRPM3	459C>T	A153A	0	1	N.R.	0	synonymous
	4023G>A	S1341S	13	28	rs3739776	—	SNP, synonymous
KLF9	459C>T	V153V	0	1	N.R.	0	synonymous
SMC5	916G>A	V306I	37	4	rs1180116	—	SNP, nonsynonymous
	922T>C	C308R	21	10	rs1180117	—	SNP, nonsynonymous
MAMDC2	62T>C	L21P	0	2	N.R.	0.02	SNP, nonsynonymous
	492C>T	T164T	0	1	N.R.	0	synonymous
	816C>T	Y272Y	11	16	rs2296772	—	SNP, synonymous
	867G>A	A289A	13	15	rs2296773	—	SNP, synonymous
	1063_1065delAAA	K355 del	11	17	rs61609258	—	(SNP) synonymous; del/ins polymorphism

TRPM3, Transient receptor potential cation channel, subfamily M, member 3; KLF9, Kruppel-like factor 9; SMC5, Structural maintenance of chromosomes protein 5; MAMDC2, MAM domain containing 2; dbSNP, registration number of database of SNP (http://www.ncbi.nlm.nih.gov/SNP/)

[a]Allele frequency was calculated from 188 chromosomes of 94 individuals.