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ORIGINAL ARTICLE

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

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The Kabuki syndrome (KS, OMIM 147920), also known as the 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 have enabled us to detect fine copy number alterations. In 17 patients with KS, molecular karyotyping was carried out with GeneChip 250K NspI array (Affymetrix) and Copy Number Analyser for GeneChip (CNAG). It showed seven copy number alterations, three deleted regions and four duplicated regions 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, namely, transient receptor potential cation channel, subfamily M, member 3 (*TRPM3*), Kruppel-like factor 9 (*KLF9*), structural maintenance of chromosomes protein 5 (*SMC5*) and MAM domain containing 2 (*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 show the causative genes for KS, but the locus of 9q21.11-q21.12, in association with a cleft palate, may contribute to the manifestation of KS in the patient. As 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.
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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 assumed 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 diagnosed in a variety of ethnic groups since 1981.[3–7] Some works have ruled out several loci; for example, 1q32–q41, 8p22–p23.1 and 22q11, as candidates for KS.[8–13] A study of array-based comparative genomic hybridization (CGH) showed a disruption of the *C20orf133(MACROD2)* gene by ~250 kb deletion in a patient with KS,[14] but the following mutation screening for the gene failed to find a pathogenic base change within exons in 19 other 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 features were not typical for KS.[16] To date, no concordant specific lesion has been

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found by whole-genome scan with array CGH in a 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, enables us to detect submicroscopic pathogenic copy number alterations, which were undetectable even by BAC array CGH.[19,20] As not a few MCA/MR syndromes are because of chromosomal copy number aberration, we hypothesize that some sort of microdeletion/microduplication causes KS. Herein, we report the results of molecular karyotyping in 17 patients using GeneChip 250K array and those of 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 the aforementioned 18 patients. The diagnoses of KS were confirmed by experts of clinical genetics, although written permission for the use of facial photographs in publications was not obtained. These Japanese patients showed a normal karyotype at a 400-band level, and were earlier 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 at Nagasaki University.

Molecular karyotyping

DNA oligomicroarray hybridization, using the GeneChip Human Mapping 250K Nsp Array (Affymetrix, Santa Clara, CA, USA), was carried out for 18 patients with KS, following the provided protocol (Affymetrix). Data were analyzed using GTYPE (GeneChip Genotyping Analysis Software) to detect copy number aberration and visualized using CNAG (Copy Number Analyser for GeneChip) version 3.[21] References for non-paired analysis of CNAG were chosen from eight unrelated individuals of HapMap samples from the Affymetrix website (http://www.affymetrix.com/support/). The resolution of this procedure was estimated as ~30–100 kb. CNAG version 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 PCR (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 was carried out using a second derivative max method. 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 the 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); and *CETN1* (for patient K34). The primer sequences of these genes are available in the online supplementary file. Internal control diploid genes were *OAZ2* and *USP21*. Primer sets of the control genes for genomic DNA were selected from the Real Time PCR Primer Sets website (http://www.realtimeprimers.org/). The control genes were confirmed to have no copy number variants on the Database of Genomic Variants (DGV) updated on 26 June 2008 (http://projects.tcag.ca/variation/). BLAST searches confirmed all primer sequences specific for the gene.

Samples were analyzed in triplicate in a 384-well format in a 10 μl final volume containing about 2 ng genomic DNA, 0.5 μM forward primer, 0.5 μM reverse primer, 0.1 Units TaKaRa ExTaq HS version (TaKaRa, Kyoto, Japan), 1× PCR buffer, 200 μM dNTP and 0.5 μM SYTO9. The amplification conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 45 cycles of

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	NR	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	NR	NR
5q21.2-q21.3	K22	1	281 kb	104301325	104581898	NR	Variation_3568
9q21.11-q21.12	K16	1[a]	1.27 Mb	71760296	73031176	*TRPM3, KLF9, SMC5, MAMDC2*	NR
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]	NR
4q12	K5	3	104 kb	54251599	54355281	NR	NR
8q11.21	K7	3	171 kb	50641101	50812548	NR	Variation_2751, 3731, 8601, 37765
10p15.2-p15.1	K5	3	142 kb	3663600	3805292	NR	NR
13q31.1	K6	3	72 kb	82451568	82523728	NR	NR
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	NR	NR
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

Abbreviations: CN, copy number; DGV, Database of Genomic Variants; NR, no registration 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]

denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 15 s The data were analyzed using LightCycler 480 Basic Software (Roche Diagnostics) and the melting curve was checked to eliminate non-specific 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 version 3.1 (Applied Biosystems). Genomic sequences were retrieved from the UCSC genome browser (assembly: March 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 the online supplementary file. Resultant electropherograms were aligned using ATGC version 3.0 (Software Development, Tokyo, Japan) and inspected visually to find DNA alterations.

In silico analysis

Relations among deleted genes were assessed using 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] The novel synonymous base substitutions found in the mutation screening were examined for their potential activation of the 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).

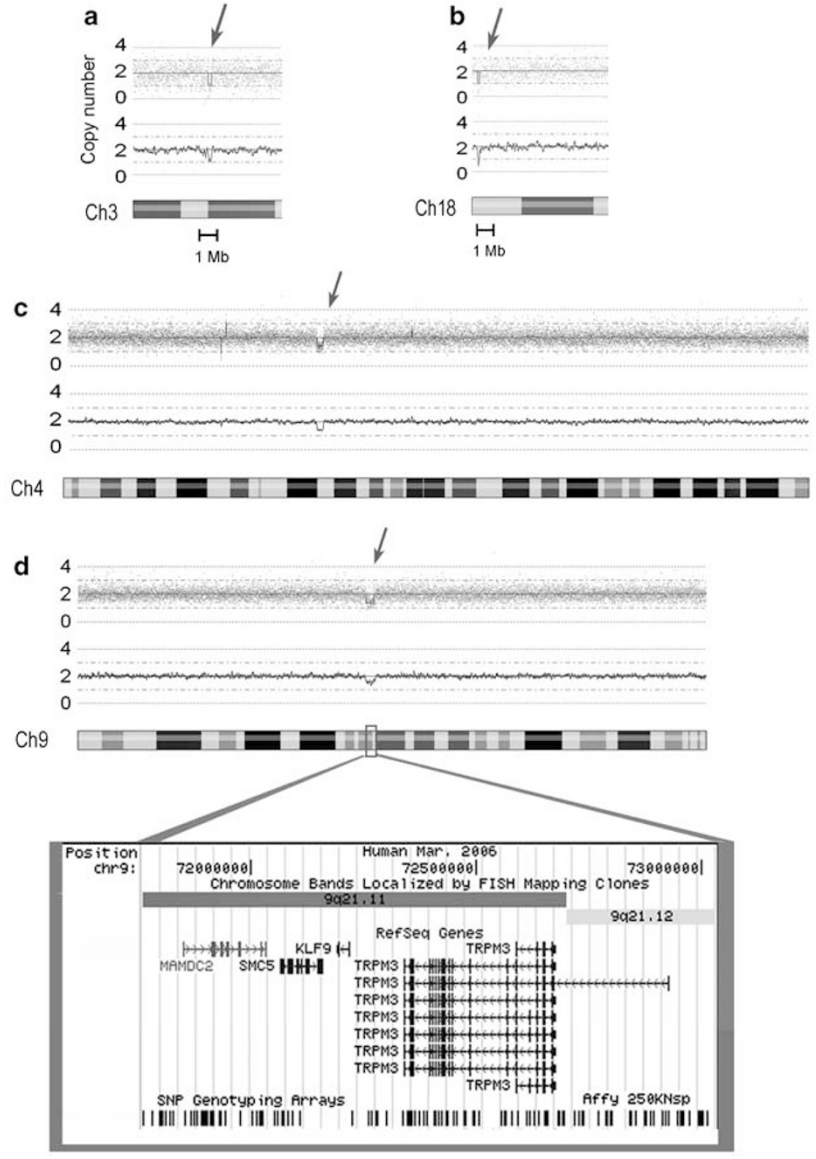

Figure 1 Chromosome view of Copy Number Analyser for GeneChip (CNAG) analysis. Each dots represent fluorescent intensity on each single-nucleotide polymorphisms (SNP) probe of GeneChip 250K NspI array (Affymetrix). Solid lines indicate copy number analyzed with CNAG. Arrows show detected deletions. (**a**) Chromosome (Ch) 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 the *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 University of California Santa Cruz 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 the Database of Genomic Variants updated 26 June 2008. FISH, fluorescent *in situ* hybridization.

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; that is, a single-nucleotide polymorphism (SNP) call rate of 82.51% and a quality control performance detection rate of 74.09%, probably because of DNA degradation during long-term storage. The other patients showed high call rates, enough for copy number analysis (SNP call rate of 90.07–97.72% and detection rate of 91.52–99.77%). We identified nine deleted regions, the lengths of which were between ~35 kb and ~1.27 Mb, and nine duplicated regions, of lengths between ~72 and ~495 kb, in the 17 patients analyzed (Table 1). As for the nine duplications detected, five of them were concordant to several observed gains in DGV, and four of them in each patient did not contain any known genes.

It is interesting that 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: transient receptor potential cation channel, subfamily M, member 3 (*TRPM3*), Kruppel-like factor 9 (*KLF9*), structural maintenance of chromosomes protein 5 (*SMC5*) and MAM domain containing 2 (*MAMDC2*) (Figure 1d). The deletion of 3p26.2 (~205 kb in patient K9, Figure 1a) had involved a non-coding exon of the *SUMF1* gene. The deletion of 18p11.32 (~35 kb in patient K34, Figure 1b) containing the *CETN1* gene had one registration in DGV as Variation_5044, which described only one observed loss and 14 observed gains in 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-pathological deletions with as many registrations as observed losses in DGV.

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

As a consequence of this copy number analysis, we considered the next four genes as candidate genes for KS: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2*.

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 SNP 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 the GeneSplicer program, but no activation of the cryptic splice site was predicted. Although 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; that is, DNA repair (*SMC5*) and mRNA transcription regulation (*KLF9*).

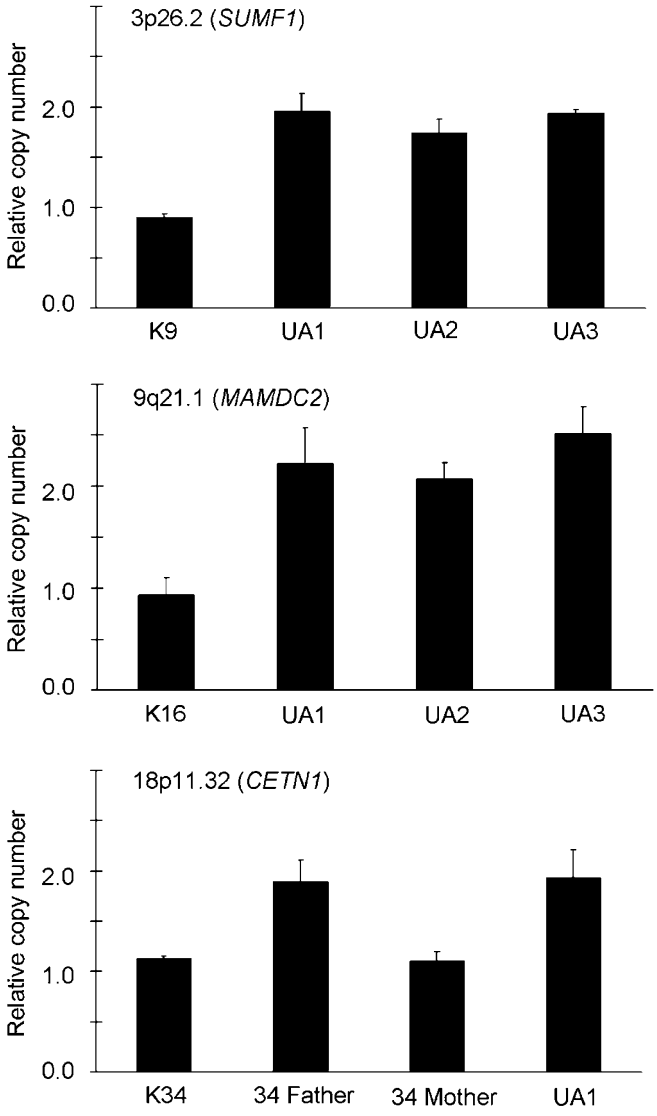

Figure 2 Validation of deletion with quantitative PCR (qPCR). qPCR confirmed a loss of one copy in each patient: *SUMF1* at 3p26.2 for patient K9; *MAMDC2* at 9q21.1 for patient K16; *CETN1* at 18p11.32 for patient K34. The deletion of patient K34 was inherited from his unaffected mother. UA, unaffected individual. Error bars, s.d.

DISCUSSION

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

The deletion in patient K16, with a length of ~1.27 Mb at 9q21.11–q21.12, harbored four known genes: *TRPM3*, *KLF9*, *SMC5* and *MAMDC2* (Figure 1d). Unfortunately, her parents' DNAs were unavailable, but the region is unlikely to be a copy number variant (CNV) because it has not been known as CNV in DGV; moreover, the deletion was not found in 95 normal Japanese individuals using qPCR.

As mutation screening in the 41 patients with KS showed no pathogenic base substitution in these genes, we cannot state that

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	NR	0	Synonymous
	4023G>A	S1341S	13	28	rs3739776	—	SNP, synonymous
KLF9	459C>T	V153V	0	1	NR	0	Synonymous
SMC5	916G>A	V306I	37	4	rs1180116	—	SNP, non-synonymous
	922T>C	C308R	21	10	rs1180117		SNP, non-synonymous
MAMDC2	62T>C	L21P	0	2	NR	0.02	SNP, non-synonymous
	492C>T	T164T	0	1	NR	0	synonymous
	816C>T	Y272Y	11	16	rs2296772	—	SNP, synonymous
	867G>A	A289A	13	15	rs2296773	—	SNP, synonymous
	1063_1065 delAAA	K355 del	11	17	rs61609258	—	(SNP) synonymous; del/ins polymorphism

Abbreviations: *KLF9*, Kruppel-like factor 9; *MAMDC2*, MAM domain containing 2; *SMC5*, structural maintenance of chromosomes protein 5; SNP, single nucleotide polymorphism; *TRPM3*, transient receptor potential cation channel, subfamily M, member 3; 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.

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 the PANTHER classification suggested that the three genes were associated with developmental biology, such as mRNA transcription regulation. Moreover, the 1.27 Mb region of 9q21 was included in an earlier reported candidate locus of cleft lip/palate by meta-analysis of linkage analysis.[24] Patient K16 actually had velopharyngeal insufficiency because of a submucous cleft palate. Therefore, it is reasonable to consider that the deleted genes cooperated with the 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 was it 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 62 other 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 on KS on deletion/duplication detected using oligonucleotide array and mutation screening of the coding genes within the region. One limitation of this study is its resolution. As a matter of course, a higher resolution array can detect smaller genomic rearrangements, which were undetectable in the same patient, as we showed here compared with an earlier study of BAC array CGH.[18] Although SNP probes are useful to examine loss of heterozygosity as a collateral evidence in deletions, unevenly distributed probes of the SNP array have a disadvantage for CNV detection. As various platforms on oligonucleotide array have developed, higher resolution platforms will have to be applied to search tiny genomic rearrangements in patients with KS. Another limitation is that we assumed that a single copy number change caused KS. It remains to be elucidated whether CNV association[25] contributes towards manifestations of KS. If further investigation with refined array technologies cannot 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 in the non-coding genomic regions.

In summary, we applied molecular karyotyping with GeneChip 250K array to detect copy number aberrations in 17 patients with KS and screened 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 as various array platforms have the potential to specify genomic alterations for KS.

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