Lack of *C20orf133* and *FLRT3* mutations in 43 patients with Kabuki syndrome in Japan

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Abbreviations: CNP, copy-number polymorphism; DGV, Database of Genomic Variants; FLRT3, fibronectin leucine rich transmembrane protein 3; KS, Kabuki syndrome; NCBI, National Center for Biotechnology Information; OMIM, Online Mendelian Inheritance in Man; SNP, single nucleotide polymorphisms; UCSC, University of California Santa Cruz, UTR, untranslated region

Background: Kabuki syndrome (KS) is a rare multiple congenital anomalies/mental retardation syndrome characterized by a peculiar facial appearance. Recently, Maas et al. reported that exon 5 of the *C20orf133* gene at 20p12.1 was disrupted by a 250-kb de novo microdeletion in a patient with KS and screened for mutations in *C20orf133* and *FLRT3* (a nested gene within intron 3 of *C20orf133*) in 19 additional patients with KS, but they failed to detect such mutations or deletions in any of the other patients.

Objective: To examine contribution of the two genes to KS, we performed mutation analysis, deletion assay and copy number analysis among Japanese patients with KS.

Methods: Direct sequencing of *C20orf133* and *FLRT3* and quantitative real-time PCR at the exon 5 region of *C20orf133* were performed in 43 patients with KS. Copy number changes at 20p12.1 were analyzed by GeneChip 250K Nsp array among 18 patients with KS.

Results: Direct sequencing of these genes did not show any pathogenic nucleotide changes in the patients. Quantitative PCR indicated that none of the 43 patients had the deletion at the region. Although copy number analysis at 20p12.1 among the 18 patients detected ~100 kb deletion in one patient within intron 5 of *C20orf133* and ~30 kb duplication within intron 4 of *C20orf133* in another patient, neither of them contain any exon.

Conclusion: These findings in most Japanese patients do not support the working hypothesis that the *C20orf133* and/or *FLRT3* are the causative gene for KS.

Kabuki syndrome (KS, OMIM 147920), also known as Niikawa-Kuroki syndrome, is a multiple congenital anomalies/mental retardation syndrome characterized by a peculiar facial appearance, skeletal abnormalities, joint hypermobility, dermatoglyphic abnormalities, postnatal growth retardation, recurrent otitis media and occasional visceral anomalies. Although some works have ruled out several loci from the candidacy for KS, any putative disease gene loci or candidate genes remain unidentified.

In a recent issue of the *Journal of Medical Genetics*, Maas et al. reported that exon 5 of the *C20orf133* gene at 20p12.1 was disrupted by a 250-kb de novo microdeletion in a patient with KS and they also screened for mutations in *C20orf133* and *FLRT3* (a nested gene located within intron 3 of *C20orf133*) in 19 additional patients with KS, but failed to detect such mutations or deletions in any of them.[1]. It remains unclear whether the two genes are responsible for the pathogenesis of KS, and if so, how frequently the deletion at the locus is found in KS patients. Herein we describe the results of a deletion assay for the exon 5 in *C20orf133* and a mutation analysis of *C20orf133* and *FLRT3* among 43 patients with KS in Japan. In addition, we also show the results of a copy number analysis at 20p12.1 by Human Mapping 250K Nsp Array among 18 patients with KS in Japan.

Ethics approval for this study was obtained from the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University. The subjects studied consisted of 43 patients (20 girls and 23 boys) with KS from Japan for mutation analysis and a deletion assay, and of 18 patients (9 girls and 9 boys) with KS for copy number analysis. Genomic DNA was isolated by the standard method. Genomic sequences were retrieved from the UCSC genome browser. GenBank accession numbers of NCBI of *C20orf133* and *FLRT3* are NM_080676 and NM_198391, respectively.

C20orf133 and *FLRT3* were directly sequenced. Polymerase chain reaction (PCR) primers used for the two genes were those described by Maas et al..[1], but some primers did not work in our laboratory, we newly designed the primes. Direct sequencing of *C20orf133* and *FLRT3* did not show any pathogenic nucleotide changes in the 43 patients. Observed five nucleotide changes in the patients were all found in normal Japanese individuals as well. That is, a first substitution, c.173C>T (p.T58I) in *C20orf133*, already registered in the database of SNPs as rs2990505, was identified in 14 patients (12 heterozygous and two

homozygous status). A second substitution, c.1069T>C (p.S357P) in *C20orf133*, not registered in the database, was observed in 10 patients (heterozygous status) and in 35 of 159 normal Japanese individuals (29 heterozygous and six homozygous status). A third substitution, g.14257801T>C in exon 2 (5'UTR exon) of *FLRT3*, found in nine patients (heterozygous status), was registered as rs761998. A fourth substitution, c.765A>G (p.Q255Q) in *FLRT3*, not registered in the database, was found in one patient (heterozygous status) and in three of 137 normal Japanese individuals (heterozygous status). The last nucleotide change, heterozygous deletions of three nucleotides, g.14257934_14257936delCAG in exon 2 (5'UTR exon) of *FLRT3*, not registered in dbSNP, was found in nine patients, and in four of 81 normal Japanese individuals.

Deletion assay involving exon 5 of *C20orf133* was performed by quantitative real-time PCR on an ABI PRISM 7900HT Sequence Detection System (AppliedBiosystems, Foster City, CA, USA). *ALB* gene was chosen as a reference gene.[2], which had no copy-number polymorphism (CNP) in the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/). Primers and fluorogenic probes were designed with the assistance of Primer Express v1.5 (AppliedBiosystems). Primer sequences are available on request. The quantitative PCR indicated that none of the 43 patients had any copy number changes involving *C20orf133* exon 5. The average quotient and standard deviation of the target/reference genes in patients was 1.090 ± 0.124 with a range of 0.934-1.291 ±0.025-0.426, and the control persons as well (data not shown).

According to the DGV (Last updated: Nov 29, 2007), a ~368-kb deletion (chromosome 20, nucleotide numbers (nt) 14,606,364-14,974,100 bp), involving exon 5 of *C20orf133* (nt 14,613,489-14,613,605), has been reported in one of 506 unrelated healthy Northern German and 270 HapMap individuals (registered as Variation_9315, a normal loss).[3]. Thus, it is possible that the 250-kb deletion at 20p12.1 in a KS patient reported by Maas et al..[1] was a rare copy-number variation. However, some CNPs have a possibility to play more important roles in human phenotypic variations than SNPs.[4, 5]. For instance, Balikova et al. reported a unique novel syndrome caused by the amplification of large genomic regions, ~750-kb at cytoband 4p16, known to be copy number variation.[6]. Therefore, we must be careful to check copy number changes for an MCA/MR syndrome.

To search particular microdeletion/duplication at cytoband 20p12.1, we performed copy number analysis among 18 patients with KS by DNA oligomicroarray hybridization using the GeneChip® Human Mapping 250K Nsp Array (Affymetrix, Santa Clara, CA, USA). Data at the target region were analyzed using GTYPE (GeneChip Genotyping Analysis Software), CNAT (GeneChip Chromosome Copy Number Analysis Tool) and Partek Genomic Suite v6.3 (Partek Inc., St. Louis, MO, USA). Two copy number changes were found among 18 patients. Neither of them was registered in the DGV, but they were less likely pathogenic because both of them were within intronic sequences. That is, a ~100-kb deletion within intron 5 of *C20orf133* was detected in one patient (patient 3 in figure 1). Its physical positions and log2 ratios were: chromosome 20, nt 15,000,514 bp (LogR -0.4136), 15,014,439 (-0.4586), 15,034,442 (-0.4831), 15,066,513 (-0.4519), and 15,102,706 (-0.4330). In another patient (patient 14 in figure 1), a ~30-kb region was suggested as duplication and the region was located within intron 4 of the *C20orf133*. The physical positions and log2 ratios were: chromosome 20, nt 14,527,943 bp (LogR 0.4113), 14,553,038 (0.4443), 14,557,957 (0.4204) and 14,563,924 (0.3878). The other 16 patients with KS did not show significant copy number changes at the region. Thus, particular copy number changes at the region were not detected in these patients with KS.

In summary, we performed a mutation analysis for *C20orf133* and *FLRT3*, a deletion assay for exon 5 of *C20orf133* in 43 patients with KS and a copy number analysis by DNA oligomicroarray among the 18 patients with KS in Japan. These studies did not reveal pathogenic alterations in the patients. Thereby, our findings unfortunately could not support the working hypothesis that the *C20orf133* and/or *FLRT3* were the causative gene in most Japanese KS patients.

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REFERENCES

1 Maas NM, Van de Putte T, Melotte C, Francis A, Schrander-Stumpel CT, Sanlaville D, Genevieve D, Lyonnet S, Dimitrov B, Devriendt K, Fryns JP, Vermeesch JR. The C20orf133 gene is disrupted in a patient with Kabuki syndrome. *J Med Genet* 2007;44:562-9.

2 Laurendeau I, Bahuau M, Vodovar N, Larramendy C, Olivi M, Bieche I, Vidaud M, Vidaud D. TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency. *Clin Chem* 1999;45:982-6.

3 Pinto D, Marshall C, Feuk L, Scherer SW. Copy-number variation in control population cohorts. *Hum Mol Genet* 2007;16:R168-173.

4 Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, Cho EK, Dallaire S, Freeman JL, González JR, Gratacòs M, Huang J, Kalaitzopoulos D, Komura D, MacDonald JR, Marshall CR, Mei R, Montgomery L, Nishimura K, Okamura K, Shen F, Somerville MJ, Tchinda J, Valsesia A, Woodwark C, Yang F, Zhang J, Zerjal T, Zhang J, Armengol L, Conrad DF, Estivill X, Tyler-Smith C, Carter NP, Aburatani H, Lee C, Jones KW, Scherer SW, Hurles ME. Global variation in copy number in the human genome. *Nature* 2006;444:444-454.

5 Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, Redon R, Bird CP, de Grassi A, Lee C, Tyler-Smith C, Carter N, Scherer SW, Tavaré S, Deloukas P, Hurles ME, Dermitzakis ET. Relative impact of nucleotide and copy number variation

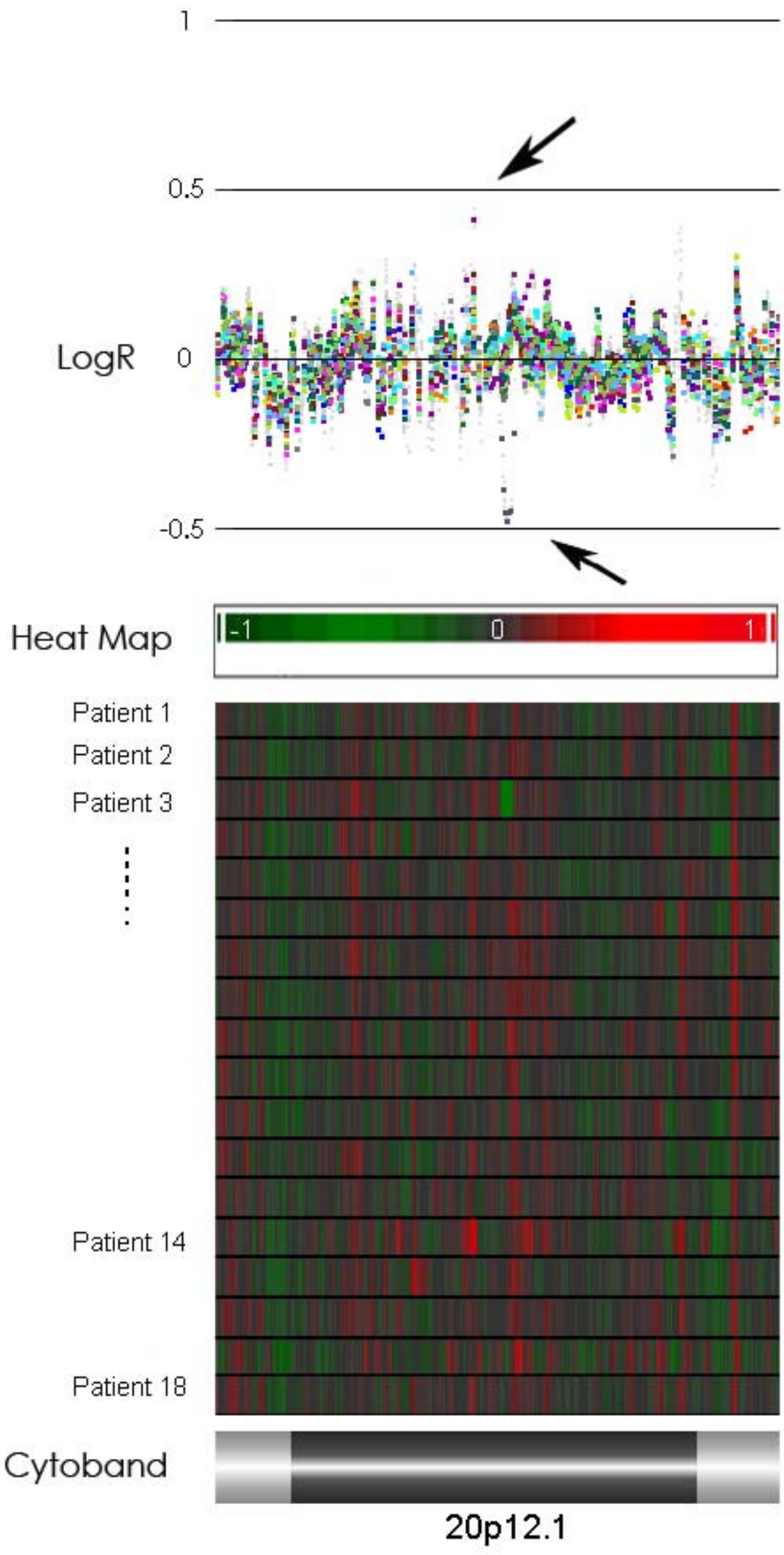
on gene expression phenotypes. *Science* 2007;315:848-853.

6 Balikova I, Martens K, Melotte C, Amyere M, Van Vooren S, Moreau Y, Vetrie D, Fiegler H, Carter NP, Liehr T, Vikkula M, Matthijs G, Fryns JP, Casteels I, Devriendt K, Vermeesch JR. Autosomal-dominant microtia linked to five tandem copies of a copy-number-variable region at chromosome 4p16. *Am J Hum Genet* 2008;82:181-7.

Legend

Figure 1 Copy number analysis at 20p12.1 among 18 patients with Kabuki syndrome.

Each patient in log2 ratio plot was showed by different colour. A bar of Heat Map representing gain for red and loss for green was divided in each patient. Deletion at intron 5 of *C20orf 133* was indicated in Patient 3. Duplication at intron 4 of *C20orf133* was suggested in Patient 14 (arrows in LogR plot; deep green or deep red in HeatMap).


1
0.5
LogR
0
-0.5
Heat Map
-1
0
1
Patient 1
Patient 2
Patient 3
Patient 14
Patient 18
Cytoband
20p12.1