

SHORT REPORT

Array based CGH and FISH fail to confirm duplication of 8p22-p23.1 in association with Kabuki syndrome

J D Hoffman, Y Zhang, J Greshock, K L Ciprero, B S Emanuel, E H Zackai, B L Weber, J E Ming

J Med Genet 2005;42:49–53. doi: 10.1136/jmg.2004.024372

Background: Kabuki (Niikawa–Kuroki) syndrome comprises a characteristic facial appearance, cleft palate, congenital heart disease, and developmental delay. Various cytogenetically visible chromosomal rearrangements have been reported in single cases, but the molecular genetic basis of the condition has not been established. A recent report described a duplication of 8p22–p23.1 in 13/13 patients.

Objective: To determine the frequency of an 8p duplication in a cohort of patients with Kabuki syndrome.

Methods: An 8p duplication was sought using two independent methods—array based comparative genomic hybridisation (aCGH) and fluorescence in situ hybridisation (FISH)—in 15 patients with a definitive clinical diagnosis of Kabuki syndrome.

Results: No evidence for a duplication of 8p was obtained by FISH or aCGH in any of the 15 patients.

Conclusions: 8p22–p23.1 duplication may not be a common mechanism for Kabuki syndrome. Another genetic abnormality may be responsible for the aetiology in many patients.

Kabuki syndrome, or Niikawa–Kuroki syndrome, comprises a characteristic facial appearance, short stature, skeletal anomalies, dermatoglyphic abnormalities, and mental retardation.^{1,2} Congenital heart disease, cleft palate, dental anomalies, and involvement of other organ systems may occur. Characteristic facial features include long palpebral fissures with eversion of the lower lids, prominent eyelashes, lateral thinning of the eyebrows, and prominent auricles. Persistent fetal fingertip pads are also commonly present. Elucidation of the molecular genetic cause of this condition has been hampered by a relative lack of familial instances or consistent chromosomal abnormalities.³ Recently, metaphase comparative genomic hybridisation (CGH) and fluorescence in situ hybridisation (FISH) have been used to detect an interstitial tandem duplication ranging between 0.83 and 3.5 megabases in chromosome region 8p22–p23.1 in a panel of patients with Kabuki syndrome.^{4,5} However, the frequency of this abnormality in the syndrome has not been clearly established. In this report, we assess for the 8p22–p23.1 duplication in a cohort of 15 subjects with a definite clinical diagnosis of Kabuki syndrome.

METHODS

Patients

All 15 subjects were evaluated in the clinical genetics centre at The Children's Hospital of Philadelphia by two of us (EHZ and JEM). The diagnosis of Kabuki syndrome was based on facial features including long eyelashes, eversion of the lower lateral eyelid, anomalous eyebrows, and a depressed nasal tip (fig 1). Three of the subjects have been described in previous

clinical reports.^{6,7} Patients provided informed consent upon enrolment in the study, which was approved by the Institutional Review Board of The Children's Hospital of Philadelphia. Signed permission to reproduce the photographs shown in fig 1 was given by the parents. All subjects had a normal karyotype (at the 500 band level or higher) and a normal subtelomeric FISH study carried out as part of the clinical evaluation. Seven subjects were male and eight were female; two were of latino ancestry and the remainder were white.

A lymphoblastoid cell line (No GM14485) from a patient who did not have Kabuki syndrome and who had an inverted duplication-deletion of 8p was obtained from the Coriell Institute for Medical Research (Camden, New Jersey, USA). The karyotype was 46,XY,del dup(8)(qter→p23.1::p23.1→p11.2). This sample was used as a positive control for the 8p duplication (clone RP11-31B7).

Fluorescence in situ hybridisation analysis

Bacterial artificial chromosome (BAC) clones were obtained from the BACPAC Resource (Oakland, California, USA) and end sequenced to verify their identity at the nucleic acid/protein core facility at The Children's Hospital of Philadelphia. FISH analysis was carried out as described previously.⁸ BACs were isolated using the Pefectprep plasmid kit (Eppendorf, Hamburg, Germany) and probes labelled with Spectrum Red or Green (Vysis Inc, Downers Grove, Illinois, USA) by nick translation. Both metaphase and interphase spreads were prepared either from peripheral blood lymphocytes or lymphoblastoid cell lines using standard methodology. Chromosomes were visualised by counterstaining with DAPI.

Array based comparative genomic hybridisation

The array based comparative genomic hybridisation (aCGH) array that we employed includes a set of 4134 BAC clones spanning the human genome at approximately a 0.92 megabase resolution.⁹ Genomic DNA was isolated using standard methods. Test genomic DNA was labelled with random hexamers and Cy3 labelled dUTP. Pooled normal control DNA was labelled with Cy5 labelled dUTP. Dual colour hybridisation was carried out and data were acquired using an Affymetrix 428 laser scanner. Data from each fluorophore were combined and the composite image imported into GenePix Pro 4.0 (Axon Instruments, Sunnyvale, California, USA). The raw fluorescence data were used to calculate a ratio for each clone (indicating the relative DNA copy number in the test sample compared with the reference sample). As an additional control, each experiment was repeated after reciprocal labelling of the samples by swapping the labelling dyes.

Abbreviations: aCGH, array based comparative genomic hybridisation; BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridisation



Figure 1 Representative subjects with Kabuki syndrome studied in this report. (A) Patient 1. (B) Patient 2. (C) Patient 3. (D) Patient 5. (E) Patient 8. (F) Patient 10. Permission to reproduce these photographs was given by the parents.

RESULTS

BAC selection

The report of the 8p duplication⁴ described a duplication of BAC clones mapping to the region between 10.04 and 12.99 megabases (Mb) on chromosome 8p (genome position as per Genome Browser, University of California, Santa Cruz (UCSC), genome.ucsc.edu, July 2003 freeze) (fig 2). We selected four of these BAC clones mapping to the 8p22–p23.1 duplication region for FISH analysis: RP11-252K12, RP11-235I5, RP11-80B8, and RP11-31B7 (table 1, fig 2). These four clones map within a 1.9 Mb region on chromosome 8p22–p23.1. In addition, BAC RP11-122N11 was noted to show three signals in interphase analysis in the report of the 8p duplication.⁴ This BAC was also analysed in our patient cohort.

Table 1 BAC clones mapping to 8p22–8p23.1 used for analysis

BAC	Base position (Mb)
RP11-252K12	10.89–11.07
RP11-235I5	11.54–11.72
RP11-297K5	11.57–11.74
RP11-80B8	11.62–11.80
RP11-31B7	12.75–12.78

Base position from UCSC Genome Browser (July 2003 freeze) (<http://genome.ucsc.edu>).

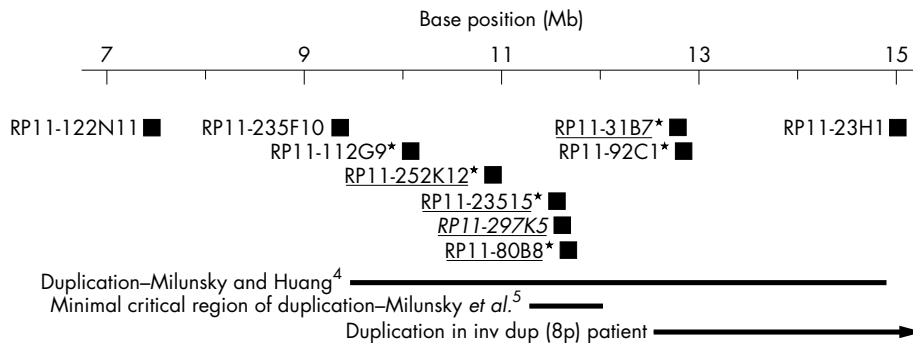


Figure 2 Chromosome region 8p22-p23.1. Genomic positions of BAC clones are indicated (UCSC Genome Browser). Starred clones were reported as duplicated by Milunsky *et al.*⁴ and clones RP11-235F10 and RP11-23H1 were reported as normal.⁴ Underlined clones were used for fluorescence in situ hybridisation in this paper; the italicised clone (RP11-297K5) was on the aCGH array. The maximum size of the 8p duplication as reported by Milunsky and Huang⁴ is indicated. The approximate location of the minimal critical region as reported by Milunsky *et al.*⁵ is denoted. The extent of the duplication in the inv dup (8p) patient used as a control is indicated by the line with the arrowhead, with the arrowhead signifying that the duplication extends further toward the centromere.

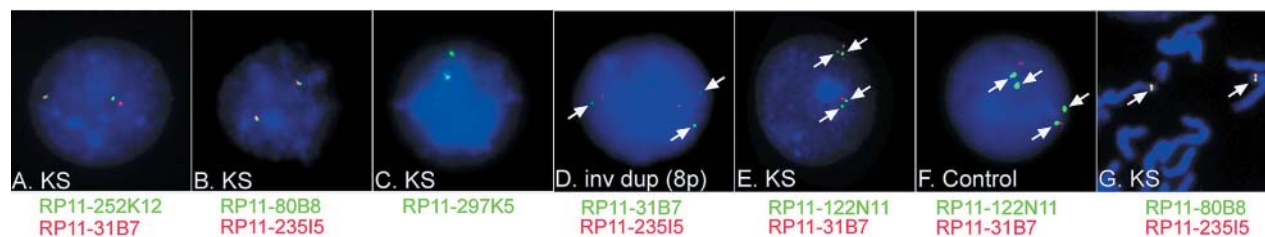


Figure 3 Fluorescence in situ hybridisation of 8p22–p23.1. BAC clones mapping to 8p22–8p23.1 served as the template for fluorescently labelled probes. Only two signals were observed in the subjects with Kabuki syndrome for each of the clones: RP11-252K12 (A), RP11-31B7 (A), RP11-235I5 (B), and RP11-80B8 (B). In addition, the clone RP11-297K5, which maps to the reported minimal critical region, also showed two signals in cases of Kabuki syndrome (C). In the Kabuki syndrome cases, only two signals for each of the clones listed in table 1 were also observed in analysis of metaphase spreads, as shown in the representative partial metaphase spread depicted of clones RP11-80B8 and RP11-235I5 (G). In the case with the inv dup (8p), RP11-31B7 showed three signals (D), consistent with the known duplication in this positive control. The clone RP11-122N11 showed four signals in samples from both Kabuki syndrome cases (E) and unaffected subjects (F).

FISH analysis of chromosome region 8p22–8p23.1

Material suitable for FISH was available in 11 of the 15 subjects. The BAC probes RP11-252K12, RP11-31B7, RP11-235I5, and RP11-80B8 were used for FISH analysis in these 11 confirmed cases of Kabuki syndrome. At least 30 interphase nuclei were examined for each probe used for each patient. FISH analysis showed that each of the four BACs had only two signals, not the three that would have been expected if a duplication were present on one chromosome 8 homolog (fig 3A, B; table 2). At least 10 metaphase spreads were also examined for each probe used for each patient, and no duplications were detected.

In order to ensure that a duplication could be readily detected, we used as a positive control a sample obtained from a subject who does not have Kabuki syndrome but who has an inverted duplication of 8p (inv dup (8p)) (karyotype 46,XY,del dup(8)(qter→p23.1::p23.1→p11.2)). This inverted duplication has been described in several individuals who do not have Kabuki syndrome and is associated with a distinct clinical phenotype that is clearly different from Kabuki syndrome. These individuals also have a deletion that involves the 8p subtelomeric region. The duplicated region in the inv dup (8p) partially overlaps but is different from the 8p duplication reported in cases of Kabuki syndrome^{10 11} (fig 2). Based on the karyotype and reports of other reported inv dup (8p) cases, it would be expected that the duplicated

material includes 8p22 and a variable amount of 8p material centromeric to genomic position 12.58 Mb on 8p22.^{11 12} The BAC RP11-31B7 maps to the duplicated region and thus was expected to be duplicated in this subject. In fact, the RP11-31B7 clone showed three signals in interphase nuclei in this positive control sample (fig 3D), consistent with a duplication of 8p22 on one of the chromosome 8 homologs. As expected, FISH with BACs RP11-252K12 and RP11-235I5 showed two signals in this case (table 2), as these BACs do not map to the duplicated region in the inv dup (8p) (fig 2). These data indicate that FISH analysis in our laboratory can detect a duplication of 8p22, if present.

FISH analysis with the BAC RP11-122N11

In the published report on the 8p duplication in Kabuki syndrome,⁴ the BAC RP11-122N11 lies outside of the duplicated region. However, it was reported to show three signals in interphase nuclei, and this was interpreted as an inversion.⁴ We undertook FISH analysis with this BAC to assess the hybridisation pattern with this clone. FISH analysis with this probe showed four signals in the cases with Kabuki syndrome that we studied (fig 3E, table 1). In order to determine the FISH pattern in individuals without Kabuki syndrome, we also carried out FISH with BAC RP11-122N11 on two samples from unaffected subjects who were not related to a person with Kabuki syndrome. Of note, a

Table 2 Copy number detected by individual BAC clones

Patient	Diagnosis	FISH					aCGH	
		RP11-252K12	RP11-235I5	RP11-80B8	RP11-31B7	RP11-122N11	RP11-297K5	
1	KS	2	2	2	2	–	2	
2	KS	2	2	2	2	4	–	
3	KS	2	2	2	2	4	2	
4	KS	2	2	2	2	–	2	
5	KS	2	2	2	2	–	2	
6	KS	2	2	2	2	–	2	
7	KS	2	2	2	2	4	2	
8	KS	2	2	2	2	4	–	
9	KS	2	2	2	2	4	–	
10	KS	2	2	2	2	–	–	
11	KS	2	2	2	2	–	–	
12	KS	–	–	–	–	–	2	
13	KS	–	–	–	–	–	2	
14	KS	–	–	–	–	–	2	
15	KS	–	–	–	–	–	2	
16	Inv dup (8p)	2	2	–	3	–	–	
17	Control	–	–	–	–	4	–	
18	Control	–	–	–	–	4	–	

KS, Kabuki syndrome; –, not determined.

similar pattern of four FISH signals was detected in the two unaffected controls as well (fig 3F). This indicates that the four signals of the RP11-122N11 probe are present in the general population, suggesting that chromosome specific, low copy repeats may be present on 8p.

aCGH analysis of the 8p22-8p23.1 region

For six of the 11 Kabuki syndrome cases studied by FISH, as well as four additional cases (10 of the total cohort of 15), we also used array based comparative genomic hybridisation (aCGH) as an independent method to test for an 8p duplication. This array can readily and reliably detect single copy changes in genome dosage.^{9, 13} The array contains the BAC clone RP11-297K5 (11.57–11.74 Mb, UCSC Genome Browser), which lies within the reported minimal duplicated region.⁵ In aCGH, the test and control genomic DNA samples are labelled with either Cy3 or Cy5, respectively. The relative intensity ratio of the signal from these dyes (Cy3: Cy5 ratio) indicates the relative copy number of a given chromosomal region. In the aCGH studies with our cohort, the Cy3: Cy5 ratio for RP11-297K5 did not vary significantly from the normal ratio of 1.0 (normal range 0.8 to 1.2) in any of the 10 Kabuki syndrome cases, indicating that the copy number was normal (table 2). We also used this clone as a FISH probe and did not detect a duplication of this BAC by this method (fig 3C). Thus the aCGH studies did not identify a duplication of 8p in the subjects examined.

DISCUSSION

Milunsky *et al* described a duplication of 8p22–8p23.1 ranging between 0.83 and 3.5 Mb in all 13 cases of Kabuki syndrome whom they studied.⁴ We used two independent platforms to assess for this duplication: aCGH and FISH. In contrast to the reported findings, we failed to detect the reported 8p22–p23.1 duplication in any of a cohort of 15 cases of Kabuki syndrome that we studied by FISH, aCGH, or both. Our data indicate that an 8p duplication was not present in this set of patients. This suggests that the 8p duplication may not be a common finding in Kabuki syndrome. Of note, a recent study described FISH analysis of 26 Japanese and two Thai cases with typical features of Kabuki syndrome, and this study also failed to detect the reported 8p22–p23.1 duplication.¹⁴

In the report of the 8p duplication,⁴ the clone RP11-122N11 was found to give three FISH signals in the cases of Kabuki syndrome and their mothers. We found that the BAC clone RP11-122N11 gave four signals in interphase FISH analysis both in the Kabuki syndrome cases and in unaffected controls. Of note, this BAC contains segmental duplication sequences, based on the UCSC Genome Browser (April 2003 freeze). The RP11-122N11 clone has only been mapped by FISH (UCSC Genome Browser), and the precise genomic location of this clone has not been unequivocally determined. Thus it is possible that this clone hybridises to low copy repeat sequences that are present in more than one region of the chromosome. Alternatively, duplications of the genomic sequence corresponding to this clone may be present in the general population, as four hybridisation signals were also seen in samples from unaffected individuals who were not related to a person with Kabuki syndrome. The genomic organisation of 8p22–p23.1 is quite complex, with several low copy repeat elements, and the finding of multiple signals of RP11-122N11 is a reflection of the complexity of the underlying genomic structure.

The reason for the discrepancy between our findings and those of Milunsky and Huang⁴ is not clear at present. The cases studied in our cohort had facial features that were characteristic of Kabuki syndrome, including long eyelashes, eversion of the lower lateral eyelid, anomalous eyebrows, and a depressed nasal tip. It is possible that there are clinical

differences between the subjects described with the duplication and those in our cohort. The cases in whom the 8p duplication was detected⁴ may represent a clinical subset. The presence of segmental duplications in the 8p22–p23.1 region may also complicate FISH analysis if the BAC clones used for FISH contain these repeat sequences. In addition, FISH may be susceptible to experimental artefacts owing to asynchronous replication of the chromosomal homologs, differences in BAC preparation, or other factors that could lead to the discrepant findings. Further investigations into the aetiology of Kabuki syndrome will be required to determine the predominant genetic cause of this condition.

ACKNOWLEDGEMENTS

We thank Sonya Babar for technical assistance. We thank the families and the Kabuki Syndrome Network for their participation. This work was supported in part by funding from the Penn Genomics Institute (to JEM) and from the Abramson Family Cancer Research Institute (to BLW). Support was also provided from the Mental Retardation and Developmental Disabilities Research Center at the Children's Hospital of Philadelphia (NIH grant HD26979).

Authors' affiliations

J D Hoffman, Y Zhang, K L Ciperro, B S Emanuel, E H Zackai, J E Ming, Division of Human Genetics, Department of Pediatrics, The Children's Hospital of Philadelphia and The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

J Greshock, B L Weber, Abramson Family Cancer Research Institute, The University of Pennsylvania

Competing interests: none declared

Correspondence to: Dr Jeffrey E Ming, The Children's Hospital of Philadelphia, Abramson Research Building, Room 1002, 3615 Civic Center Boulevard, Philadelphia, PA 19104, USA; jeming@mail.med.upenn.edu

REFERENCES

- 1 **Niikawa N,** Matsuura N, Fukushima Y, Ohsawa T, Kajii T. Kabuki make-up syndrome: a syndrome of mental retardation, unusual facies, large and protruding ears, and postnatal growth deficiency. *J Pediatr* 1981;**99**:565–9.
- 2 **Kuroki Y,** Suzuki Y, Chyo H, Hata A, Matsui I. A new malformation syndrome of long palpebral fissures, large ears, depressed nasal tip, and skeletal anomalies associated with postnatal dwarfism and mental retardation. *J Pediatr* 1981;**99**:570–3.
- 3 **Matsumoto N,** Niikawa N. Kabuki make-up syndrome: a review. *Am J Med Genet* 2003;**117C**:57–65.
- 4 **Milunsky JM,** Huang XL. Unmasking Kabuki syndrome: chromosome 8p22–8p23.1 duplication revealed by comparative genomic hybridization and BAC-FISH. *Clin Genet* 2003;**64**:509–16.
- 5 **Milunsky JM,** Maher TA, Huang XL. Refinement of the Kabuki syndrome critical region. American College of Medical Genetics, Annual Clinical Genetics Meeting, Kissimmee, FL, 2004; <http://www.spltrak.com/ACMG/CategoryAbstracts/cat3.html>.
- 6 **Ming JE,** Russell KL, Bason L, McDonald-McGinn DM, Zackai EH. Coloboma and other ophthalmologic anomalies in Kabuki syndrome: distinction from CHARGE association. *Am J Med Genet* 2003;**123A**:249–52.
- 7 **Ming JE,** Russell KL, McDonald-McGinn DM, Zackai EH. Autoimmune disease in Kabuki syndrome. *Am J Med Genet* (in press).
- 8 **Shaikh TH,** Kurahashi H, Saitta SC, O'Hare AM, Hu P, Roe BA, Driscoll DA, McDonald-McGinn DM, Zackai EH, Budarf ML, Emanuel BS. Chromosome 22-specific low copy repeats and the 22q11.2 deletion syndrome: genomic organization and deletion endpoint analysis. *Hum Mol Genet* 2000;**9**:489–501.
- 9 **Greshock J,** Naylor TL, Margolin A, Diskin S, Cleaver SH, Futreal PA, DeJong PJ, Zhao S, Liebman M, Weber BL. 1-Mb resolution array-based comparative genomic hybridization using a BAC clone set optimized for cancer gene analysis. *Genome Res* 2004;**14**:179–87.
- 10 **Florida G,** Piantanida M, Minelli A, Dellavecchia C, Bonaglia C, Rossi E, Gimelli G, Croci G, Franchi F, Gilgenkrantz S, Grammatico P, Dalpra L, Wood S, Danesino C, Zuffardi O. The same molecular mechanism at the maternal meiosis I produces mono- and dicentric 8p duplications. *Am J Hum Genet* 1996;**58**:785–96.
- 11 **Giglio S,** Broman KW, Matsumoto N, Calvari V, Gimelli G, Neumann T, Ohashi H, Voullaire L, Larizza D, Giarda R, Weber JL, Ledbetter DH, Zuffardi O. Olfactory receptor-gene clusters, genomic-inversion polymorphisms, and common chromosome rearrangements. *Am J Hum Genet* 2001;**68**:874–83.
- 12 **Sugawara H,** Harada N, Ida T, Ishida T, Ledbetter DH, Yoshiura K, Ohta T, Kishino T, Niikawa N, Matsumoto N. Complex low-copy repeats associated

- with a common polymorphic inversion at human chromosome 8p23. *Genomics* 2003;**82**:238–44.
- 13 **Mosse Y**, Greshock J, King A, Khazi D, Weber BL, Maris JM. Identification and high-resolution mapping of a constitutional 11q deletion in an infant with multifocal neuroblastoma. *Lancet Oncol* 2003;**4**:769–71.
- 14 **Miyake N**, Harada N, Shimokawa O, Ohashi H, Kurosawa K, Matsumoto T, Fukushima Y, Nagai T, Shotelersuk V, Yoshiura K, Ohta T, Kishino T, Niikawa N, Matsumoto N. On the reported 8p22-p23.1 duplication in Kabuki make-up syndrome (KMS) and its absence in patients with typical KMS. *Am J Med Genet* 2004;**128A**:170–2.

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