ment. The array was hybridized in the presence of Cot-1 DNA and blocking agents for 40 hours at 65°C, washed, and scanned by GenePix 4000B (Axon Instruments). Data were extracted w Agilent Feature Extraction software version 9 using default settings for CGH. Statistically significant aberrations were determined using the ADM-II algorithm in the CGH analytics version 3.5 (Agilent Technologies). Breakpoints were defined as the start and stop location of the first and last probes, respectively, included in the algorithmically determined region of deletion.

FISH Analysis

Metaphase or prometaphase chromosomes were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes according to standard techniques. RP11 BAC clones were selected from in silico library build 2006 and were purchased from Invitrogen (Table 1).

FISH analyses using the combination with two BAC clones were performed according to the following method. After hardening chromosome slides at 65°C for 150 min, they were denatured in 70% formamide containing 2× standard saline citrate (SSC) at 70°C for 2 min, and then dehydrated at −20°C in ethanol. BAC clone DNA was extracted using GenePrepStar PI-80X (Kurabo) and labeled with SpectrumGreen TM-11-dUTP or SpectrumOrange TM-11-dUTP (Vysis, Downers Grove, IL) by nick translation and then denatured at 70°C for 5 min. The probe-hybridization mixture was applied to the chromosomes, which were incubated at 37°C for 16 h. Slides were washed twice in 50% formamide containing 2× SSC at 43°C for 15 min, then in 2× SSC for 5 min, 1× SSC for 5 min, 0.1% Triton X-100 containing 4× SSC for 5 min with shaking, 4× SSC for 5 min and 2× SSC for 5 min. Slides were then mounted in antifade solution (Vector Laboratories, Burlingame, CA) containing 4′,6-diamino-2-phenylindole (DAPI). Fluorescence photomicroscopy was performed as described previously (Miyake *et al.*, 2003).

Mutation Screening of *HIP1* and *YWHAG* Coding Regions

All exons of *HIP1* and *YWHAG* (21 and 3 total exons, respectively) were amplified by PCR using originally designed primers derived from the neighboring intronic sequences of each exon (Supporting Information Tables S2 and S3), according to standard methods. All amplicons were subjected to direct sequencing using the BigDye terminator cycle sequencing kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Sequencing results were analyzed using the 3130xl Genetic Analyzer (Applied Biosystems).

Zebrafish Maintenance

Adult zebrafish (Danio rerio) were maintained at 28.5°C under 14-hr light/10-hr dark cycle conditions. Fertilized eggs from natural crosses were collected a few minutes after spawning and cultured at 28.5°C in water containing 0.006% NaCl and 0.00025% methylene blue. Embryos were staged according to morphology and hours post-fertilization (hpf) as described (Kimmel *et al.*, 1995). N-Phenylthiourea was added to culture water at a final concentration of 0.003% to avoid pigmentation of larvae.

Sequence Analyses of Zebrafish *hip1* and *ywhag1*

The nucleotide sequence of *hip1* cDNA has been predicted from the genomic information of zebrafish. The cDNA corresponding to the ORF of *hip1* was experimentally cloned by RT-PCR using primers designed based on the predicted sequence. The 5′- and 3′-UTRs of *hip1* were also cloned by RACE. To design antisense MOs against *ywhag1*, the 5′-UTR in our zebrafish strain, Michigan, was cloned using 5′ RACE. Then, the cDNA sequence, Accession #NM_21302, was obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). In addition, fragments surrounding the exon-intron junctions of *ywhag1*, including the putative exon 1/intron 1 and intron 1/exon 2 junctions, were amplified by PCR using genomic DNA as a template and primers corresponding to the zebrafish whole-genome shotgun scaffolds Zv7_NA122 and Zv7_NA727. Total RNA was extracted from embryos 24-hpf using the RNeasy Mini Kit (QIAgen), and cDNA was synthesized using the Omniscript RT Kit (QIAgen) according to the manufacturer's instructions. Extraction of genomic DNA from adult zebrafish was performed as described (Westerfield, 1995). All PCR amplification reactions were performed using KOD plus DNA polymerase (Toyobo, Osaka, Japan). For 5′ RACE, the 5′-RACE Core Set (Takara, Otsu, Japan) was used. The sequences of the primers used in this study are shown in Supporting information Table S4.

Morpholinos and Microinjection

MOs were purchased from Gene Tools (Philomath, OR). For gene knockdown of *hip1*, two nonoverlapping translation-inhibiting MOs (*hip1*-MO1, *hip1*-MO2) were used. For *ywhag1*, one translation-inhibiting MO (*ywhag1*-MO1) and one splice-inhibiting MO (*ywhag1*-spMO) were employed. As a negative control, a standard control MO was used. The sequences of these MOs are shown in Supplemental Table S4. MOs were resuspended at 10 μg/μl in Danieau solution (5 mM HEPES, pH 7.6, 58 m*M* NaCl, 0.7 m*M* KCl, 0.4 m*M* $MgSO_4$, and 0.6 m*M* $Ca(NO_3)_2$) and stored at −20°C. Microinjections were performed as described (Razzaque *et al.*, 2007).

Preparation of Zebrafish Head Sections

Zebrafish embryos at the 72 hpf were fixed with 4% paraformaldehyde in PBS at 4°C overnight. The fixed embryos were serially dehydrated in ethanol, soaked in xylene, and embedded in Paraplast Plus embedding medium (McCormick Scientific, St. Louis, MO) under microscopic observation. Specimens were cut into serial sec-

tions (7 μm) and stained with Mayer's hematoxylin and eosin solutions.

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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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Keywords: 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 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).

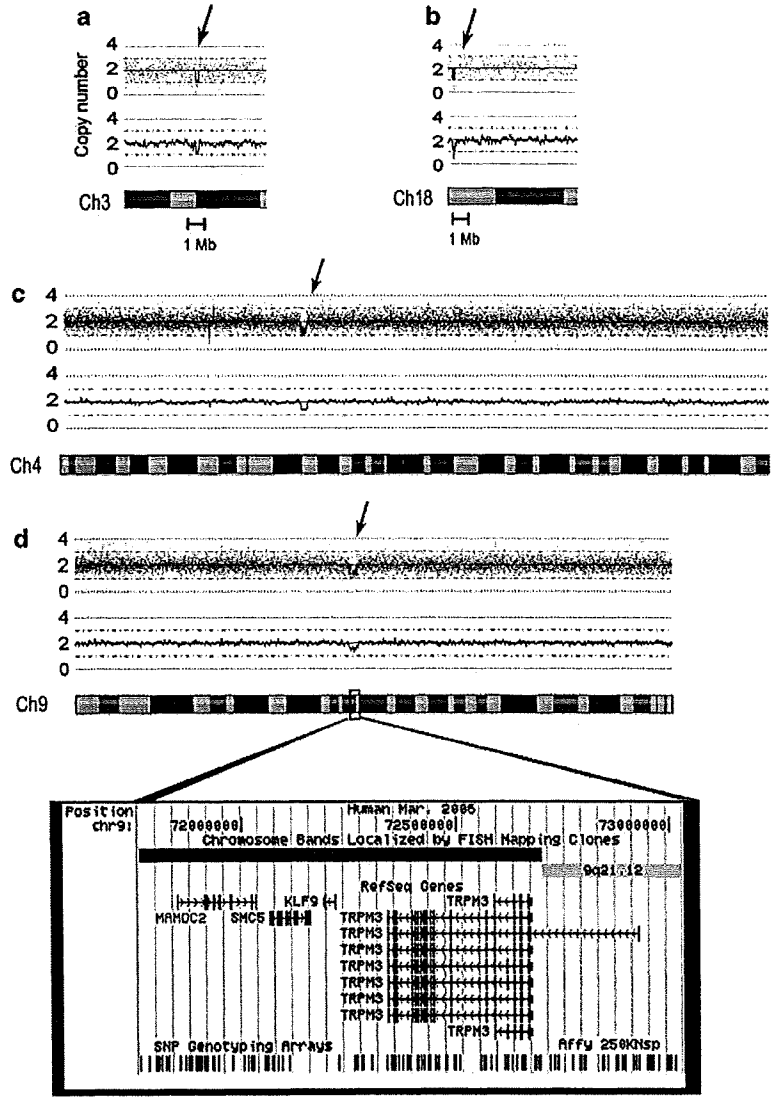

Figure 1 Chromosome view of Copy Number Analyser for GeneChip (CNAG) analysis. Each dots represent fluorescent intensity on each single-nucleotide polymorphisms (SNP) probe of GeneChip 250K NspI array (Affymetrix). Solid lines indicate copy number analyzed with CNAG. Arrows show detected deletions. **(a)** 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*).

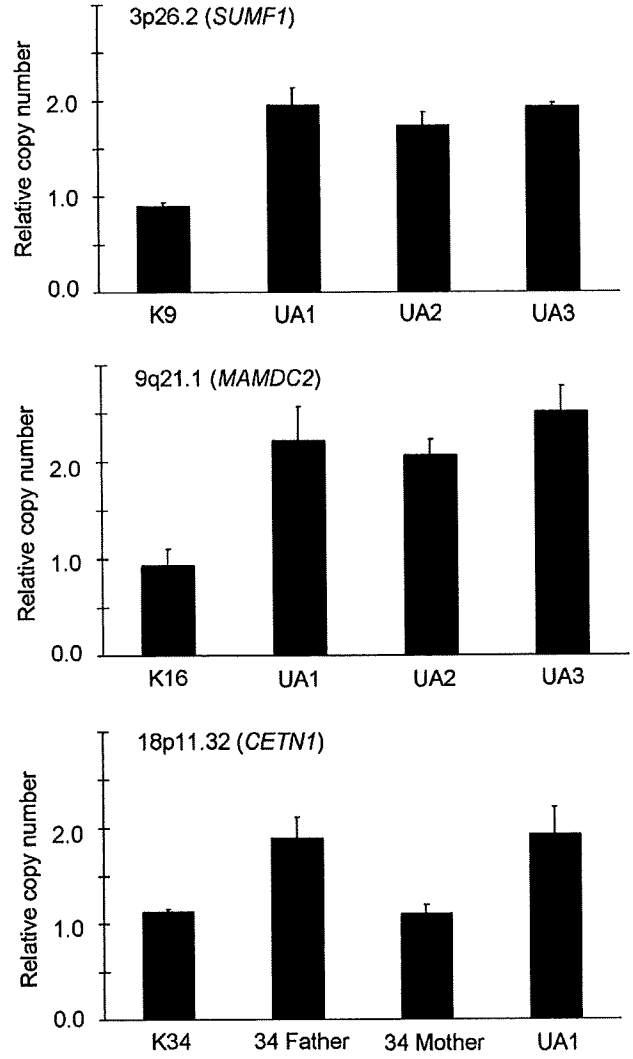

Figure 2 Validation of deletion with quantitative PCR (qPCR). qPCR confirmed a loss of one copy in each patient: *SUMF1* at 3p26.2 for patient K9; *MAMDC2* at 9q21.1 for patient K16; *CETN1* at 18p11.32 for patient K34. The deletion of patient K34 was inherited from his unaffected mother. UA, unaffected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

A case of Baraitser–Winter syndrome with unusual brain MRI findings: Pachygyria, subcortical-band heterotopia, and periventricular heterotopia

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Abstract

Baraitser–Winter syndrome (BaWS) is characterized by iris coloboma, ptosis, hypertelorism, and mental retardation; it is a rare multiple congenital anomaly or a mental-retardation syndrome of unknown etiology. Patients suffering from this syndrome have been also found to show brain anomalies such as pachygyria, subcortical-band heterotopia (SBH), and hippocampal malformations; therefore, these anomalies have been included in the phenotypic spectrum of this syndrome. We report the case of a Japanese boy suffering from BaWS; the patient's brain magnetic resonance imaging scan revealed pachygyria, SBH, and periventricular heterotopia. However, the results of the genome-wide array comparative genomic hybridization did not reveal any chromosomal rearrangements.

Keywords: Baraitser–Winter syndrome; Pachygyria; Subcortical-band heterotopia; Periventricular heterotopia

1. Introduction

Baraitser–Winter syndrome (BaWS)—characterized by iris coloboma, ptosis, hypertelorism, and mental retardation—was first described by Baraitser and Winter in 1988 [Online Mendelian Inheritance in Man (OMIM) No. 243310] (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=243310) [1]. Subsequently, some BaWS patients were found to develop pachygyria with or without subcortical-band heterotopia (SBH), both of which were revealed by brain magnetic resonance imaging (MRI) [2,3]. The genetic mechanism underlying BaWS has not been elucidated; however, 2 patients with BaWS exhibited a pericentric inversion of chromosome 2: inv(2)(p12q14) [4,5]. Ramer et al. speculated that the *PAX8* gene (OMIM No. 167415), which maps to 2q12–q14, a site that coincides with the distal breakpoint of the inversion, plays an etiological role in BaWS [6].

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Here, we report the case of a Japanese boy with BaWS whose brain MRI scans showed abnormalities; however, the genome-wide array comparative genomic hybridization (aCGH) did not reveal any chromosomal rearrangements.

2. Case report

Our patient was a 12-month-old Japanese boy who was the only child of unrelated parents; he did not have a family history of any serious diseases or allergies. Fetal ultrasonography performed at 20 weeks of gestation revealed nuchal cystic hygroma. He was born at 41 weeks of gestation. At the time of birth, he weighed 3554 g [standard deviation (SD), +1.32], his length was 48.5 cm (SD, −0.24), and the circumference of his head was 33.4 cm (SD, +0.07). Physical examination revealed that the child had epicanthal folds, ptosis, hypertelorism, a broad nasal bridge, a thin upper lip, low-set malformed ears, and a webbed neck (Fig. 1). Ophthalmologic examination revealed iris coloboma, microphthalmia, and microcornea. Cardiac ultrasonography revealed patent ductus arteriosus, mitral-valve prolapse, mitral regurgitation, tricuspid-valve prolapse, and tricuspid regurgitation, and renal ultrasonography revealed bilateral hydronephrosis. Brain MRI performed at 5 days revealed diffuse pachygyria, SBH, and periventricular heterotopia (Fig. 2A and B). We also noted incomplete hippocampal inversion (figure not shown). The patient was diagnosed with BaWS by 2 dysmorphologists. G-banding chromosome analysis of the peripheral lymphocytes showed that the patient had a normal karyotype (46, XY). Fluorescence in situ hybridization was performed using a *LIS1* probe; both chromosomes generated appropriate signals. The bacterial artificial chromosome (BAC)-aCGH analysis (comprising 4219 clones with 0.7-Mb resolution for genome-wide analysis) did not reveal any abnormalities (data not shown) [7]. When the child was 8 days old, he suffered from convulsions, which were ameliorated with administration of phenobarbital. The electroencephalography (EEG) revealed age-appropriate centroparietal dominant theta–delta background activity without any distinctive epileptic discharges. At 4 months of age, the patient's auditory brainstem response was within the normal range. Eczema and eosinophilia were noted during the neonatal period; these conditions exacerbated at 8 months (white blood cell count, $57.7 \times 10^3/\mu l$; eosinophil proportion, 54.0%) and improved after administration of oral prednisolone.

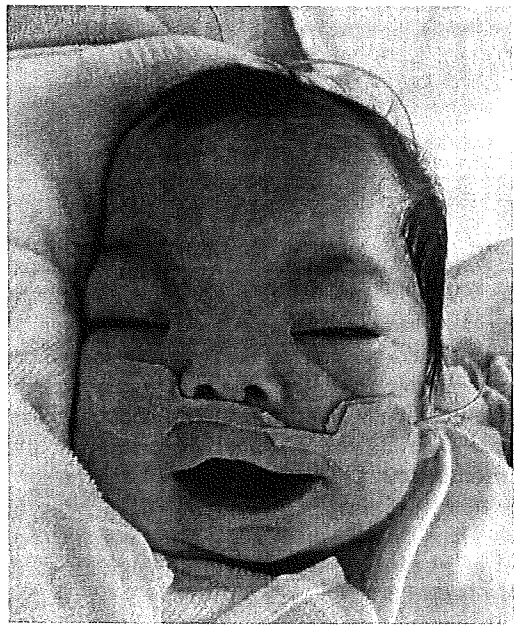

Fig. 1. The patient at 11 months. Epicanthal folds, ptosis, hypertelorism, a broad nasal bridge, long hypoplastic philtrum, and the thin vermilion border of the upper lip can be observed.

At 12 months of age, the patient weighed 5804 g (SD, −3.8), his length was 62.0 cm (SD, −5.0), and the circumference of his head was 43.0 cm (SD, −2.2). He was unable to hold up his head and had not yet started to utter words. A physical examination revealed the absence of hepatosplenomegaly. A neurological examination revealed muscle hypotonia, poor head control, normal deep-tendon reflexes, and positive Babinski sign. The patient did not exhibit blink reflexes on exposure to acoustic or visual stimuli, and he showed poor visual tracking; however, exposure to tactile stimuli evoked smiles. During the follow-up examination, a brain MRI scan showed that there were marked changes in the white matter; this observation indicated the enlargement of the perivascular space (Fig. 2C). EEG revealed centroparietal dominant theta background activity, intermittent occipital dominant alpha–beta activities, 14-Hz sleep spindles, relatively poor interhemispheric synchronization, and no distinctive epileptic discharges. The levels of organic acid and mucopolysaccharides in the urine were within normal ranges.

3. Discussion

In this report, we have described a patient exhibiting the characteristic features of the BaWS phenotype [1]. In patients with BaWS, developmental delay is inevitable, albeit with some variations [1–6]. The symptoms manifested in our patient seemed to correspond to those observed in the most severe case (patient 2 in the study by Ramer et al.) [6]. The severity of developmental delay can reflect the degree of brain malformation. Both patient 2 and our patient showed classical lissencephaly, which causes severe developmental delay. Marked eosinophilia, which was observed in our patient, has not been reported to be associated with BaWS. Thus far, there

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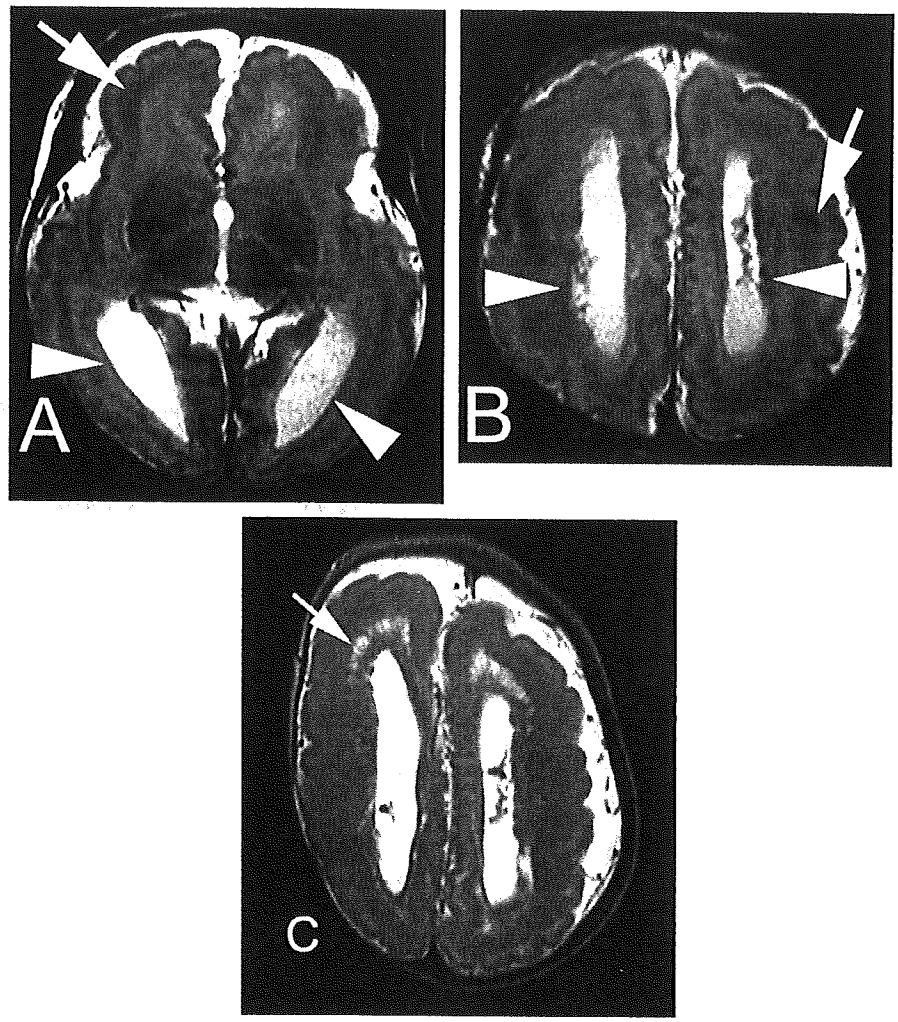

Fig. 2. Axial T2-weighted brain MRI scans (repetition time (TR)/echo time (TE), 4000/120 ms; 1.5 T scanner) at 5 days (A and B) and 11 months (C). (A and B) Diffuse pachygyria with SBH (arrows) and periventricular heterotopia (arrow heads) can be observed. (C) Marked changes are observed in the white matter; the affected regions are isointense to the regions containing the cerebrospinal fluid (arrow). The feather-like configurations are suggestive of an enlarged perivascular space.

have been very few reports on BaWS, and no BaWS-specific genetic or biological markers have been identified; therefore, it is unclear whether the marked eosinophilia is incidental or pathognomonic.

A case of BaWS with pachygyria and SBH has already been reported; however, BaWS with periventricular heterotopia, which was observed in our patient, has not yet been reported [2,3]. Patients with these conditions may show impaired neuronal migration. It is well known that mutations in the *LIS1* or *DCX* genes can cause pachygyria or SBH, and that abnormalities in the *FLNA* gene can result in periventricular heterotopia (also known as periventricular nodular heterotopia); however, the brain MRI scan of our patient differed from that of the patients with the abovementioned conditions. To date, the combination of pachygyria, SBH, and periventricular heterotopia has not been reported to be associated with known cortical malformations induced by gene abnormalities [8,9]. Enlargement of the perivascular space has been reported to be associated with BaWS [2,3]. The marked changes in the white matter, observed in our patient at 12 months, were probably due to this enlargement; however, the mechanisms underlying this enlargement are still unknown. The hippocampal malformation observed in our patient has also been reported to be related to BaWS; this malformation could have developed primarily from cortical malformations [2].

In a previous study, 2 patients with BaWS were found to have a pericentric inversion in chromosome 2: inv(2)(p12q14). However, the exact etiology of BaWS remains unknown [4,5]. In our patient, BAC aCGH and G-banding chromosome analyses did not reveal any abnormalities. Therefore, we believe that the causa-

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tive genomic change was too minor, as discussed in Pallotta's study, to be detected by the abovementioned methods.

The combination of pachygyria, SBH, and periventricular heterotopia observed in our patient may be a key finding for clarifying the etiology of BaWS; additional case reports and advancements in genetic-analysis tools are required for achieving the same.

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特集：*小児疾患における臨床遺伝学の進歩*

大田原症候群

加 藤 光 広

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大田原症候群

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要旨

大田原症候群は，1976年に日本の大田原らによって初めて報告された乳児期早期に発症する年齢依存性てんかん性脳症であり，その多くは難治性でWest症候群に移行する最重度のてんかん症候群である．これまで大田原症候群は脳形成障害に併発する2次性疾患と考えられてきたが，大田原症候群の原因遺伝子として，2007年に*ARX*を，2008年に*STXBP1*を同定することができた．*ARX*は抑制性大脳介在ニューロンの発生に必須であり，ポリアラニン配列の伸長数に比例して発症早期化・重症化し，年齢依存性の機序を分子レベルで明らかにした．*STXBP1*はシナプス放出障害という新しいてんかん発症機序を呈示した．

Key words：大田原症候群，てんかん，介在ニューロン病，*ARX*，*STXBP1*

Ⅰ 大田原症候群の歴史と特徴

大田原症候群は，1976年に岡山大学小児科の大田原俊輔医師（後に岡山大学小児神経科初代教授）らによって，日本小児神経学研究会（現日本小児神経学会）機関誌の「脳と発達」に「特異な年齢依存性てんかん性脳症 The early-infantile epileptic encephalopathy with suppression-burst」として初めて報告された[1]．現在でも広く用いられている1989年の国際抗てんかん連盟による分類では，「サプレッション・バーストを伴う早期乳児てんかん性脳症」としてほぼ原著論文の題名どおりの病名が採用され[2]，2001年の新分類提案では「大田原症候群 Ohtahara syndrome」に変更され，日本人の名前を冠した疾患名が公式に認められた[3]．

大田原症候群は，大田原らが原著論文で述べるように「脳波上suppression-burstを示し，乳児期早期（生後3カ月以内）発症，頻回のtonic spasms，著しい難治性と重篤な予後を特徴とする」[1]．症例の75％は生後1カ月以内に発症し，その後75％は脳波所見がヒプスアリスミアとなりWest症候群に変容する[4]．大田原症候群の最大の特徴は覚醒・睡眠を問わず持続的に出現するサプレッション・バーストと命名された脳波所見である（図1）．

原因として，片側巨脳症やAicardi症候群，孔脳症など脳形成障害の併発が多く症候性てんかんに分類され，症例のほとんどは孤発例のため，特定の遺伝子異常が直接的に大田原症候群の原因になるとは考えられていなかった．1995年に常染色体優性夜間前頭葉てんかんの原因遺伝子が同定されてから，多数の特発性てんかんにおいて各種イオンチャネルの遺伝子異常が報告されたが，症候性てんかんの遺伝子探求は無謀と思われたかもしれない．しかし滑脳症を中心とする脳形成障害がもう一つの研究テーマの筆者にとっては，大田原症候群の遺伝子解析に着手したのは自然な流れであった．

Ⅱ *ARX*遺伝子

1 *ARX*遺伝子と介在ニューロン

*ARX*遺伝子は，1997年に三菱化学生命科学研究所の北村邦夫博士らによって新規に同定されたホメオボックス遺伝子である．発生過程のマウスの大脳（前脳）に発現し，遺伝子の構造がショウジョウバエの*aristaless*（*al*）遺伝子と高い（85％）相同性を示すことから，*aristaless*

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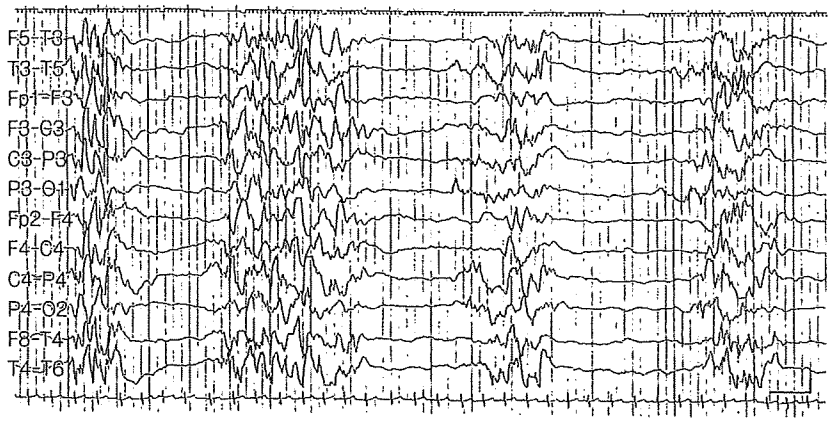

図1　大田原症候群で認められる脳波のサプレッション・バースト
不規則な徐波と棘波が混合した150～300 μVの広汎性高振幅群発が，2～3秒間隔で平坦波と交互に出現する．

related homeobox gene (*Arx*) と名付けられた．*Arx* 遺伝子欠損マウスの病理形態が，米国シカゴから報告されていたヒトの脳梁欠損と外性器異常を伴うX連鎖性滑脳症（XLAG）と類似していたことから日米共同研究が行われ，*ARX* がXLAGの原因遺伝子であることが判明した[5]．2001年9月からシカゴ大学のDobyns教授のもとに留学していた筆者は臨床症状や画像をまとめ，*ARX* 遺伝子解析を始めた．Nature Genetics誌に投稿した6日後の同誌online版に，*ARX* 遺伝子の変異が家族性精神遅滞やてんかんの原因になることが報告され[6]，筆者はてんかんの中でも点頭てんかん（West症候群）の発症率が高いことに注目した．

てんかん発作は以前から神経細胞の興奮と抑制のバランスが崩れ，興奮優位になったときに生じると考えられていた．*Arx* 欠損マウスでは抑制性大脳介在ニューロンの基底核原基での産生と移動，分化が障害され，介在ニューロン（抑制）の減少をまねく．*Arx* 欠損マウスは胎生致死のためてんかん発作は確認できないが，*ARX* 遺伝子変異をもつXLAGの患者は出生当日から難治性のけいれん発作を頻回にきたし，介在ニューロンの機能欠損を支持する[7][8]．2002年3月にフランスから3家系のXLAGの剖検例が報告され，XLAGの大脳皮質では正常な6層構造を示さず，非錐体細胞が失われ錐体細胞のみによって形成される3層構造を示した[9]．さっそくフランスの著者に連絡をとって検体をシカゴに送ってもらい，剖検例でも *ARX* 遺伝子変異を検出しマウスの結果がヒトでも当てはまることを確認した[10]．

2．West症候群

ARX の機能障害によってんかん発作が起きることを確信し，秋田大学の沢石由記夫先生から紹介された潜因性West症候群で，孤発例で初めてとなる遺伝子変異を報告した[11]．2003年9月に帰国してからも *ARX* 遺伝子解析を継続し，全国の小児神経科医との共同研究でWest症候群の解析を続け，筆者が主治医のジストニアを伴うWest症候群で第1ポリアラニンの伸長変異を認めた．ARX蛋白質にはアラニン残基が10個前後連続するポリアラニン配列が4カ所存在する（図2）．第2ポリアラニン配列には変異が好発し，*ARX* はX連鎖性精神遅滞では脆弱X症候群に次ぐ頻度である．ジストニアを伴うWest症候群では，16個のアラニン残基が連続する第1ポリアラニン配列が

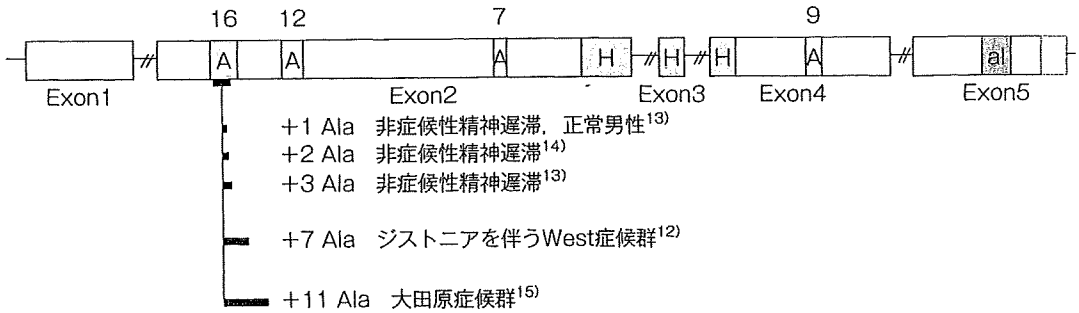

図2 *ARX*遺伝子の構造と第1ポリアラニン配列の伸長数と表現型の関係
第1ポリアラニンのアラニン残基の数が長くなるほど発症年齢は早くなり，症状も重度化する．A：ポリアラニン配列（上の数字は連続するアラニン残基の数），H：ホメオドメイン，al：アリスタレスドメイン

23個に伸びていた．同じ変異をシカゴ留学中に米国の1家系で同定し，イタリアのGuerrini教授からジストニアを伴うウエスト症候群の2家系で同じ変異を認めたことを聞いていたので，イタリア・日本・米国の症例をまとめ共同で発表した[12]．

3．大田原症候群

XLAGの患者は出生日からミオクローヌスを主体とする激しいけいれん発作をきたし生涯難治であるが，West症候群の特徴であるシリーズ形成するスパスムや脳波でヒプスアリスミアを呈する症例はない．そのなかで，1例のみがサプレッション・バーストを呈していた[7]．サプレッション・バーストが大田原症候群の特徴であり，大田原症候群がWest症候群に移行し，*ARX*遺伝子変異がWest症候群の原因であることを知っていれば，当然*ARX*遺伝子は大田原症候群の候補遺伝子となる．

2006年2月に北海道大学の斉藤伸治先生から小陰茎を伴う大田原症候群の男児例が紹介された．XLAGの主症状の一つは外性器低形成であり，事前に*ARX*遺伝子が原因の可能性が高いと考えはしたが，解析結果は第1ポリアラニン配列内に33塩基の重複，すなわちアミノ酸レベルでは連続するアラニンが16残基から27残基に増加する過去に報告がない変異だった．イタリアと米国にも同変異はみつかっておらず，1例のみでは大田原症候群の原因と断言するには弱いと考えていた．2006年の御用納めの日に，岩手医科大学の亀井淳先生から紹介いただいた患者で，北海道大学の症例とまったく同じ第1ポリアラニン配列の伸長変異を認めた．後で知った臨床症状は，大田原症候群のほかに，小陰茎，大脳白質萎縮による脳室拡大，脳梁菲薄化，髄鞘化遅延を認め非常に類似していた．

*ARX*遺伝子の第1ポリアラニン配列が2〜3個のアラニン残基だけ増えるより短い伸長変異では，West症候群や脳形成障害を伴わない非症候性精神遅滞をきたす[13)14)]．つまり*ARX*遺伝子ポリアラニン配列の伸長数が増えるほど症状は重度化し，発症時期は早期化することが判明した（図2）．ポリグルタミン配列の伸長変異や非翻訳領域の反復配列でも伸長数と重症度，発症時期が相関し，3塩基の繰り返しが多いことからトリプレット・リピート病と称されている．*ARX*遺伝子のポリアラニン配列は単純な3塩基の反復ではなく，同一家系内での反復配列の段階的な増加は認められないが，複数の患者を比較すると，他のトリプレット・リピート病でみられる表現促進現象（anticipation）様のパターンが明らかである．発症年齢の違いが遺伝子変異による表現促進現象で説明されたことは予想以上の成果だった[15)16)]．*ARX*遺伝子の

変異はほかにも水無脳症や脳梁欠損，ジストニアを伴う精神遅滞など多彩な表現型を呈し，「介在ニューロン病」としてまとめられる[7)17)]．

III *STXBP1* 遺伝子

ARX 遺伝子の同定と前後して横浜市立大学環境分子医科学の松本直通教授から，難治性てんかんの患者でゲノム解析を行い候補遺伝子を絞ったので，どのような症例の検体を集めればよいか相談された．症例の臨床的特徴は，新生児早期発症，頻回のけいれん発作，非てんかん性ミオクローヌス，治療抵抗性であったが，脳波は非典型的サプレッション・バーストとの記載で，早期ミオクロニーてんかんもしくは大田原症候群に類似していた．常染色体遺伝子ということで筆者が *ARX* を，横浜で新規候補遺伝子を解析する共同研究を行うことになった．

当初はやはり検体がなかなか集まらなかったが，前述した大田原症候群の *ARX* 遺伝子変異の論文が公表されてから少しずつ解析依頼が増えてきた．自身が出張している病院の重症心身障害病棟でも，点頭てんかんと病名がついていた患者の古いカルテを読み直してみたところ，前医の紹介状に「生後 36 日目の脳波でヒプスアリスミア（数秒から十数秒の棘波の介在する広汎性高振幅徐波バーストとそれに続く数秒間の electrical silence が交互にみられ，かつ spike の focus は一定せず hypsarrhythmia というべき所見の報告を得た）」との記載を認めた．所見はヒプスアリスミアではなく，まさにサプレッション・バーストと考えられるが，診断されたのは 1970 年，大田原症候群が報告される 6 年前であり，サプレッション・バーストということば自体がない時代であった．30 数年後でも通用する詳細な診療記録を目にして，正確に所見を記載することの大切さを改めて学ぶことができた．

最終的に 13 例の大田原症候群を追加して解析し，4 例で *STXBP1* 遺伝子にミスセンス変異を認めた．すでに父親が亡くなっていた 1 例を除き両親には変異を認めない新生変異であることを確認した上で，変異蛋白質の構造解析と機能解析が行われた．その結果，変異蛋白質は正常に比べ 3 次構造の折りたたみが不十分であり，熱に対する安定性が低下し，シナプス小胞の開口放出に必須の STX1A 蛋白質との結合が低下していることが明らかになった．*STXBP1* 遺伝子変異によりシナプス小胞の開口が障害され，神経伝達物質の放出が抑制されると推測される．てんかんの分子病態として主にイオンチャネルの機能解析が行われてきたが，*STXBP1* はシナプス小胞の開口放出障害という新しい発症機序を呈示した[18)]．論文は 2008 年 2 月 8 日に Nature Genetics 誌に投稿され，5 月 11 日に online 版で公表され，12 日付各新聞朝刊で紹介された．

おわりに

筆者が大田原症候群の原因遺伝子同定にかかわれたのは，たくさんの共同研究者と主治医および患者ご家族の協力のおかげであり，心から感謝したい．年齢依存性てんかん性脳症で原因が判明したのはごく一部であり，West 症候群や大田原症候群でも原因不明の症例が多い．今後も研究を継続し治療に結びつけたい．

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Case report

Analysis of the hypothalamus in a case of X-linked lissencephaly with abnormal genitalia (XLAG)

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Abstract

X-linked lissencephaly with abnormal genitalia (XLAG) is characterized by lissencephaly, absent corpus callosum and ambiguous genitalia. We examined hypothalamic dysfunctions in a XLAG case with a novel mutation of the ARX gene, and performed immunohistochemical evaluation of the diencephalons in autopsy brain. A 1-year-old boy showed intractable epilepsy, persistent diarrhea and disturbed temperature regulation. This case had abnormalities in circadian rhythms and pituitary hormone reserve test. He died of pneumonia. The globus pallidus and subthalamic nucleus was not identified, and the putamen and thalamus were dysplasic. The suprachiasmatic nucleus was absent. A few neurons immunoreactive for vasopressin seemed to form the ectopic supraoptic-like nucleus. The diencephalons were disturbed differently in each sub-region, and the changes may be related to various hypothalamic dysfunctions.

Keywords: Lissencephaly; XLAG; ARX gene; Hypothalamus; Diencephalon

1. Introduction

X-linked lissencephaly with abnormal genitalia (XLAG) is characterized by lissencephaly, absent corpus callosum and ambiguous genitalia [1]. Kitamura et al. identified loss-of-function mutations in the ARX gene in individuals affected with XLAG [2]. XLAG boys show intractable epilepsy of neonatal onset, severe diarrhea and early death [3]. Altered hypothalamic functions are speculated, but the detailed endocrine analysis has rarely been done in XLAG patients [4]. We reported that immunohistochemistry can characterize the intermingled diencephalon in cases of alobar holoprosencephaly [5]. We attempted to correlate the immunohistochemical findings in the diencephalon with data in endocrine tests in an autopsy case of XLAG with a novel mutation of ARX gene.

2. Case report

A boy was the first child born to non-consanguineous healthy parents. He was born at 40 weeks after uneventful pregnancy. Apgar scores were 4 and 7 at 1 and 5 min, respectively. Birth weight was 2552 g, length 54.5 cm, and occipito-frontal circumference 33.5 cm. He showed respiratory distress at birth, and clonic convulsion in the upper limbs with myoclonus at eyelids. Micropenis, hypospadias and cryptorchism were observed. He

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showed systemic muscle hypotonia. Blood test for TORCH complex and various metabolic tests in the urine, blood and cerebrospinal fluid were unremarkable. Magnetic resonance imaging (MRI) of the brain revealed total absence of the corpus callosum and lissencephaly with a posterior-to-anterior gradient of severity, consisting of frontal pachygyria and posterior agyria (Fig. 1a). The cerebral cortex was thick, and the basal ganglia was hypoplasic. The posterior lobe of pituitary gland was detected (Fig. 1b). Chromosome test showed 46, XY in G-band and ish Yp11.3 (SRYx1) in SRY FISH. He demonstrated a deletion of 4 bases (980–983delAACA) in exon 2 of the ARX gene, a novel mutation [6], but his parents had no abnormalities in the ARX gene. His convulsions were refractory. His body temperature fluctuated and often showed an abnormal dip. He repetitively suffered from bacterial infections, and developed intractable diarrhea. At 16 months, he died of pneumonia.

2.1. Endocrine examination

At neonate, concentration of hypocretin-1 was normal (230 pg/ml) (220–360 pg/ml in controls) in the cerebrospinal fluid. Serum level of testosterone was low (68 ng/dl) (100–200 ng/dl in controls), whereas that of free triiodothyronine (3.0 pg/ml) and free thyroxin (fT4, 1.35 ng/dl) was unremarkable. Subcutaneous administration of adrenocorticotropic hormone (ACTH) increased serum level of cortisol from 2.8 to 48.8 μg/dl, and intravenous administration of corticotrophin-releasing hormone (CRH) could induce an elevation of serum levels of ACTH (38.3–48.1 pg/ml) and cortisol (1.4–3.3 μg/dl). At 1 month, pituitary hormone reserve was tested with simultaneous intravenous administration of CRH (250 $\mu g/m^2$, body surface), thyrotropin-releasing hormone (10 μg/kg) and luteinizing hormone-releasing hormone (3 μg/kg). Thyroid stimulating hormone (TSH) showed prolonged reaction. Gonadotropins demonstrated high value before stimulation and showed prolonged reaction. Serum levels of prolactin and ACTH were high before provocation, and the former was increased after stimulation, whereas the latter seems to be unchanged. Since insufficient secretion of CRH in the hypothalamus was speculated, replacement therapy with hydrocortisone started at 2 months. Serum level of fT4 decreased without increased serum level of TSH, and became less than 0.9 ng/dl at 3 months (0.97–1.79 ng/dl in controls), and replacement therapy with levothyroxine sodium was initiated. Serial recording of rectal temperature was performed for 1 month to assess circadian rhythm of core body temperature at 3 months. The core body temperature ranged from 34 to 38 °C, lacking definite circadian rhythm (Fig. 2a). At 4 months, wakefulness–sleep circadian rhythm was monitored with an actogram by means of Actiwatch (Mini Mitter) for 7 consecutive days. Actogram identified the wake and sleep states in a day, indicating the presence of ultradian rhythm, whereas there seemed to be no circadian rhythm (Fig. 2b).

2.2. Neuropathological findings

The brain weighed 270 g at autopsy, and the brain was very small. The surface of cerebrum was smooth predominantly in the occipital lobe. The olfactory bulbs and tracts were absent, the optic chiasm and pituitary infundibulum were found. The corpus callosum, anterior and posterior commissurae and Probst bundle were absent. Formalin-fixed brain was cut coronally and embedded in paraffin. Each brain section was stained with hematoxylin–eosin, Klüver–Barrera (KB), Bodian and Holzer methods. In serial sections of the diencephalon (Fig. 3a), we performed immunohistochemistry using

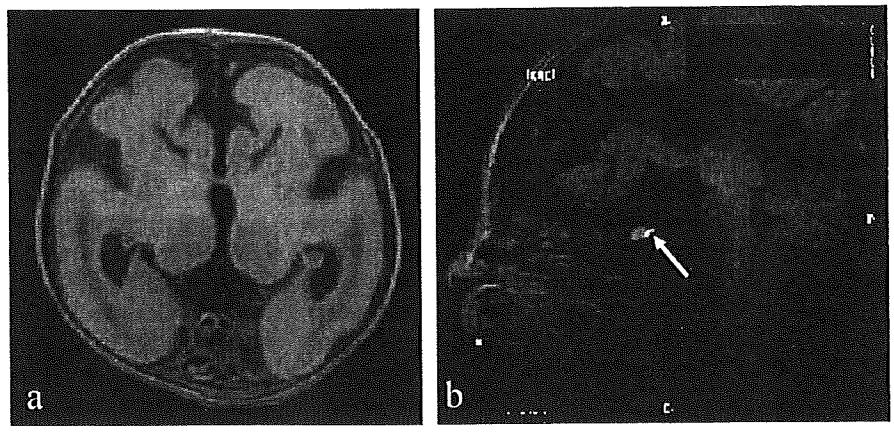

Fig. 1. Brain MRI findings at the age of 1 day. Axial view of T1-weighted image demonstrated total absence of the corpus callosum and lissencephaly, consisting of frontal pachygyria and posterior agyria (a). Sagittal view of T1-weighted image demonstrated normal high signal in the posterior lobe of the pituitary grand (arrow) (b).