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Original Article

1p36 deletion syndrome associated with Prader–Willi-like phenotype

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Abstract ***Background***: 1p36 deletion syndrome is one of the most common subtelomeric deletion syndromes, characterized by moderate to severe mental retardation, characteristic facial appearance, hypotonia, obesity, and seizures. The clinical features often overlap with those of Prader–Willi syndrome (PWS). To elucidate the phenotype–genotype correlation in 1p36 deletion syndrome, two cases involving a PWS-like phenotype were analyzed on molecular cytogenetics.

Methods: Two patients presenting with the PWS-like phenotype but having negative results for PWS underwent fluorescence *in situ* hybridization (FISH). The size of the chromosome 1p36 deletions was characterized using probes of BAC clones based on the University of California, Santa Cruz (UCSC) Genome Browser.

Results: PWS was excluded on FISH and methylation-specific polymerase chain reaction. Subsequent FISH using the probe D1Z2 showed deletion of the 1p36.3 region, confirming the diagnosis of 1p36 deletion syndrome. Further analysis characterized the 1p36 deletions as being located between 4.17 and 4.36 Mb in patient 1 and between 4.89 and 6.09 Mb in patient 2.

Conclusion: Patients with 1p36 deletion syndrome exhibit a PWS-like phenotype and are therefore probably underdiagnosed. The possible involvement of the terminal 4 Mb region of chromosome 1p36 in the PWS-like phenotype is hypothesized.

Key words 1p36 deletion syndrome, chromosome, fluorescence *in situ* hybridization, obesity, Prader–Willi-like phenotype.

The terminal deletion of chromosome 1p36 is a newly recognized syndrome with multiple congenital anomalies and mental retardation.[1–4] The prevalence is estimated to range from 1 in 5000 to 1 in 10 000.[1,5] The most frequent clinical findings are moderate–severe mental retardation, facial characteristics including deep-set eyes and pointed chin, hypotonia, and seizures. The deletion size varies in each family and appears to be correlated with the clinical complexity as a result of haploinsufficiency of different genes,[6,7] but most breakpoints cluster at 4.0–4.5 Mb from the telomere (1pter). Some clinical manifestations of the syndrome overlap with those of Prader–Willi syndrome (PWS). Recently, a PWS-like phenotype has been described in patients with monosomy 1p36,[8] maternal uniparental disomy 14 (upd[14]mat),[9,10] and chromosome 6q16 deletion.[11] The common clinical features are global developmental delay, hypotonia, obesity, several craniofacial anomalies, hyperphagia, and behavioral problems.

Here, we describe two cases of 1p36 deletion syndrome in patients who were provisionally diagnosed with PWS, and elucidate the phenotype–genotype correlation in 1p36 deletion syndrome. The study highlights the issues regarding the overlapping clinical findings and manifestations between 1p36 deletion syndrome and PWS.

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Methods

Case reports

Patient 1

Patient 1 was the first child of healthy unrelated parents, with an unremarkable family history. The mother and father were 25 and 29 years of age, respectively, at the time of her birth. She was born at 37 weeks of gestation after an uneventful pregnancy, with a birthweight of 2360 g (–0.71 SD) and length of 46.0 cm (–0.32 SD). The patient had hypotonia and difficulty in sucking, requiring tube feeding, in the neonatal period. At age 3 her cognitive skills and motor development were moderately delayed. She crawled at 10 months, walked at 18 months, and could speak repeated words at 3 years. At age 6 the patient had hyperphagia. On physical examination at the age of 9 years, her weight was 36.9 kg (+1.16 SD), height was 129.4 cm (–0.43 SD), and occipitofrontal circumference (OFC) was 52.8 cm (+1.24 SD). The facial features included deep-set eyes associated with almond-shaped palpebral fissures, straight eyebrows, a prominent forehead, a broad and flat nasal root, and a pointed chin (Fig. 1). She

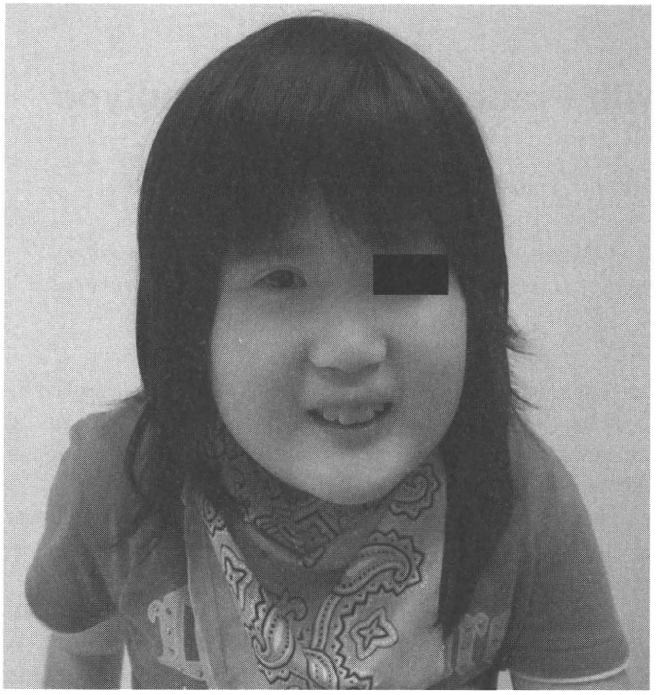

Fig. 1 Patient 1 at age 9. Note the deep-set eyes associated with almond-shaped palpebral fissures, straight eyebrows, prominent forehead, broad and flat nasal root, and pointed chin.

had small and narrow hands with a straight ulnar border and small feet with short toes. Her skin was generally hypopigmented. On initial evaluation the physical features and behavioral characteristics suggested PWS because she was given a score of 8.5 using the consensus diagnostic criteria for PWS (Table 1).[12,13]

Patient 2

Patient 2 was a boy aged 10 years, who was born at 41 weeks of gestation to non-consanguineous parents of Russian descent after an uneventful pregnancy. The mother was 21 years old and the father was 31 years old at the time of his birth. His birthweight was 2780 g (−1.19 SD) and length was 51.0 cm (+0.24 SD). He had hypotonia and difficulty in sucking during the neonatal period. His psychomotor development was apparently delayed: he walked at 19 months and could speak repeated words at 5 years. At the age of 6 years he developed generalized tonic–clonic seizures, easily controlled with valproate, but electroencephalogram and magnetic resonance imaging were normal. At school-going age he developed hyperphagia, which resulted in obesity. His mother conducted hard dietary restriction, which in turn caused malnutrition. Behavioral problems included temper outbursts and impulsivity. On physical examination at age 10 his height was 122.0 cm (−2.9 SD), weight was 24.5 kg (−1.4 SD), and OFC was 51.0 cm (−0.29 SD). He had deep-set eyes, straight eyebrows, hypopigmentation, strabismus, and a pointed chin (Fig. 2). He was given a score of 7.5 using the consensus diagnostic criteria for PWS (Table 1). Laboratory findings, including insulin-like growth factor-1 and growth hormone (GH) provocative tests indicated subnormal GH secretion. Low serum prealbumin, retinol binding protein indicated malnutrition.

Table 1 Scoring based on the diagnostic criteria for PWS[12]

	Patient 1	Patient 2
Major criteria		
Infantile central hypotonia	+	+
Infantile feeding problems/failure to thrive	+	−
Rapid weight gain between 1 and 6 years	+	+
Characteristic facial appearance	+	+
Hypogonadism: genital hypoplasia, pubertal deficiency	−	−
Developmental delay/mental retardation	+	+
Hyperphagia/food foraging/obsession with food	+	+
Cytogenetic or molecular diagnostic testing	−	−
Minor criteria		
Decreased fetal movement and infantile lethargy	−	−
Typical behavior problem	−	+
Sleep disturbance/sleep apnea	−	−
Short stature for the family by age 15 years	+	+
Hypopigmentation	+	+
Small hands and feet for height age	+	−
Narrow hands with straight ulnar border	+	−
Esotropia, myopia	−	+
Thick, viscous saliva	−	−
Speech articulation defects	+	+
Skin picking	−	−
Total scores	8.5	7.5

PWS, Prader–Willi syndrome.

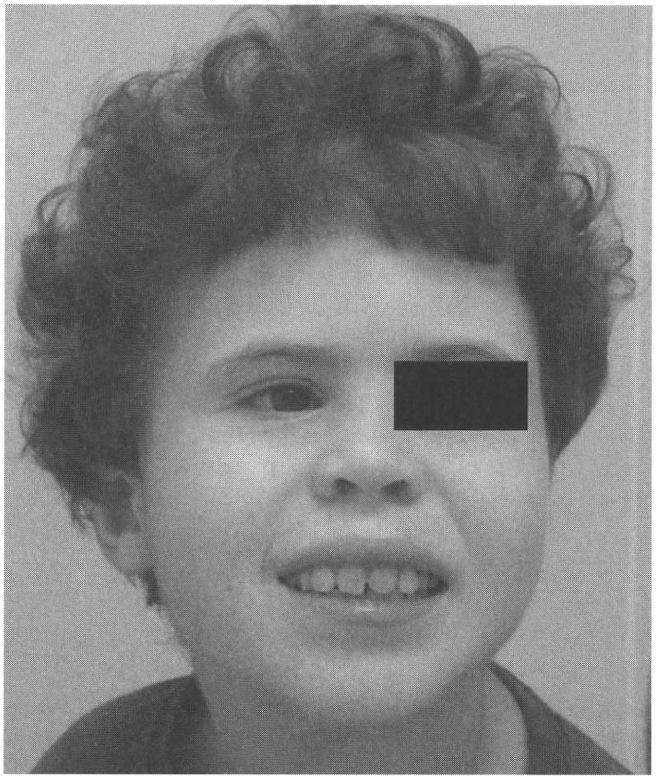

Fig. 2 Patient 2 at 10 years of age.

Molecular analysis

Genomic DNA was purified from whole blood and treated with sodium bisulfite according to the standard methods. Methylation-specific polymerase chain reaction (MS-PCR) of the *SNURF-SNRPN* exon 1 and promoter region was performed with primers described previously.[14]

Cytogenetics and fluorescence in situ *hybridization*

Cytogenetics of chromosomes from phytohemagglutinin-stimulated peripheral blood lymphocytes was performed according to the standard protocols.

Deletion screening for the PWS critical region was performed using the commercially available LSI *SNRPN* probe (Vysis; Abbot Molecular, Des Plaines, IL, USA). For screening of the terminal deletion of the short arm of chromosome 1, FISH was carried out using the probe D1Z2 mapped on 1p36.3. BAC clones were used as the probes for FISH to characterize the range of deletion; these clones were selected by the University of California, Santa Cruz (UCSC) Genome Browser from the Human March 2006 assembly (http://genome.ucsc.edu/). Bacterial stabs of the BAC clones were streaked onto Luria-Bertani plates with an appropriate antibiotic. For probes, DNA was isolated from overnight cultures with the appropriate antibiotic using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). All DNA were labeled by nick translation according to the manufacturer's instructions (Nick Translation Mix; Roche Diagnostics, Basel, Switzerland). The probes were blocked with Cot-1 DNA (Roche Diagnostics) to suppress repetitive sequences. Slides were baked at 65°C for proper aging. Chromosomes and probes were denatured on a hotplate at 75°C for 3 min and then hybridized overnight at 37°C. The slides were washed with 0.4X SSC and 0.3% NP-40 at 70°C for 2 min, washed with 0.2X SSC and 0.1% NP-40 at room temperature for 30 s, and then stained with DAPI for 3 min. Hybridization, post-hybridization washing, and counterstaining were carried out according to the standard procedures. The slides were analyzed using a completely motorized epifluorescence microscope (Leica DMRXA2) equipped with a CCD camera. Both the camera and the microscope were controlled with Leica CW4000 M-FISH software (Leica Microsystems Imaging Solutions, Cambridge, UK).

Written informed consent was obtained from the parents of both patients participating in the study, in accordance with the Kanagawa Children's Medical Center Review Board and Ethics Committee.

Results

Conventional cytogenetic analysis demonstrated a normal karyotype in both patients. FISH using a probe corresponding to *SNRPN* within the PWS region of 15q11–q13 showed no deletion. MS-PCR of chromosome 15 showed biparental methylation patterns at the *SNRPN* exon 1 region, withdrawing the diagnosis of PWS. Subsequent FISH using D1Z2 corresponding to 1p36.3 showed deletion of the region, confirming the diagnosis of 1p36 deletion syndrome in both patients.

We further applied molecular cytogenetic techniques using the BAC clones to characterize the size of the deletions. The results of these analyses on both patients are summarized in (Fig. 3). In both the patients, the deletion breakpoints were common within the chromosomal band 1p36.32 but at different regions between RP11-624G13 (4 000 095–4 178 764) and RP11-113E11 (4 366 091–4 546 558) in patient 1 and between RP11-58I24 (4 722 126–4 898 111) and RP11-71M19 (6 097 961–6 283 696) in patient 2. These analyses established the 1p36 deletions as being located between 4.17 and 4.36 Mb in patient 1 and between 4.89 and 6.09 Mb in patient 2. The parents of both patients had normal karyotype.

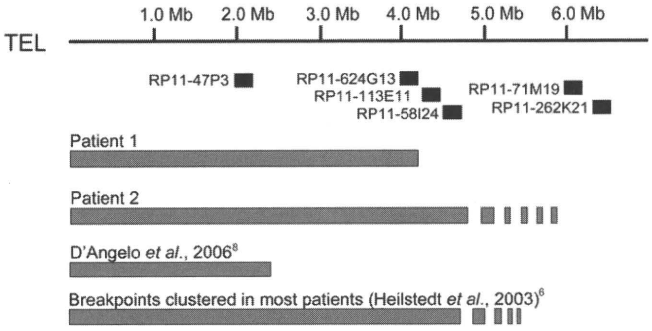

Fig. 3 Characterization of 1p36 deletion. Gray bars, deleted regions. The location of the BAC analyzed are shown according to the University of California, Santa Cruz (UCSC) Genome Browser from the Human March 2006 assembly.

Discussion

Given the clinical history and neurological features of both the patients, we arrived at a preliminary diagnosis of PWS. The facial appearance, hypopigmentation, mental retardation, feeding difficulties in the neonatal period, and hypotonia together with the characteristic behavior, including hyperphagia, were suggestive of PWS (Table 1). In children older than 3 years of age with 8 points in the consensus diagnostic criteria (4 from the major criteria), PWS should be suspected.[12,13] We were unable, however, to demonstrate deletion of the critical region of PWS (proximal long arm of chromosome 15 [15q11–q13]) and the methylation pattern of *SNRPN* exon 1 for PWS. Considering the distinctive facial features, we decided to perform FISH using D1Z2 mapped on 1p36.3, and we found a de novo deletion of this region in both patients.

Patients with 1p36 deletion syndrome have clinical features overlapping those of PWS. Furthermore, the PWS-like phenotype has been described in patients with chromosome Xq duplication,[15,16] fragile X syndrome,[17] upd(14)mat,[9,10] and 6q deletion syndrome.[11] Slavotinek *et al.* reviewed 39 patients reported to have pure 1p36 deletion, and found 2 (5.1%) with the PWS-like phenotype.[2] Using FISH and/or microsatellite markers, D'Angelo *et al.* screened 41 patients with negative results for PWS, presenting with hypotonia, developmental delay, obesity and/or hyperphagia, and behavioral problems, and detected a patient with a subtelomeric deletion of 1p.[8] Mitter *et al.* analyzed a cohort of 33 patients with low birthweight, feeding difficulties, and consecutive obesity for whom PWS was excluded on methylation analysis of *SNRPN*, and detected upd(14)mat in four of the

patients.[10] PWS is known to be one of the most common microdeletion syndromes, one of the most frequent disorders seen in genetics clinics, and the most commonly recognized genetic form of obesity.[18] Therefore additional screening on FISH with the appropriate probes combined with MS-PCR at the maternally expressed gene 3 (*MEG3*; also referred to as *GTL2* for gene trap locus 2) promoter region in patients with PWS-like phenotype should be considered for alternative diagnoses.

We determined the deletion breakpoints on FISH using the BAC clones mapped on the critical regions in both of the present patients: the breakpoints were different in both patients. D'Angelo *et al.* demonstrated that their patient with the 1p36 deletion and PWS-like phenotype had a terminal deletion of 2.5 Mb (Fig. 3); the authors suggested that the chromosomal segment 1p36.33-p36.32 is the critical region for the manifestation of obesity and hyperphagia.[8] Genotype–phenotype correlations may be useful to locate the genes responsible for several clinical features of the syndrome;[6] the degree of mental retardation is dependent on the deletion size. Heilstedt *et al.* analyzed the breakpoints in 61 patients with the 1p36 deletion, and elucidated potential critical regions for the clinical findings of facial clefts, hypothyroidism, cardiomyopathy, hearing loss, large fontanel, and hypotonia.[6] In the Battaglia *et al.* study, behavioral disorders were commonly observed (47%) in the patients with the 1p36 deletion, including self-biting of hands and wrists (30%), temper tantrums (22%), and hyperphagia (13%), overlapping the typical phenotype of PWS.[4] Reduced social interaction and severe–profound mental retardation, however, are distinct features of 1p36 deletion from PWS. Together with the present results and the D'Angelo *et al.* study, we suggest that the critical region for the PWS-like phenotype is within 4 Mb from 1pter.

In summary, the clinical features of 1p36 deletion syndrome overlap those of PWS, recognized as the PWS-like phenotype. We mapped the aberrations in two patients with the 1p36 deletion associated with the PWS-like phenotype using molecular cytogenetics. We hypothesize the possible involvement of the terminal 4 Mb region of chromosome 1p36 in the PWS-like phenotype.

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Original article

Familial brain arteriovenous malformation maps to 5p13–q14, 15q11–q13 or 18p11: Linkage analysis with clipped fingernail DNA on high-density SNP array

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ABSTRACT

Familial arteriovenous malformations (AVM) in the brain is a very rare disease. It is defined as its occurrence in two or more relatives (up to third-degree relatives) in a family without any associated disorders, such as hereditary hemorrhagic telangiectasia. We encountered a Japanese family with brain AVM in which four affected members in four successive generations were observed. One DNA sample extracted from leukocytes of the proband and ten DNA samples from clipped finger nails of other members were available. A genome-wide linkage analysis was performed on this pedigree using Affymetrix GeneCip 10K 2.0 Xba Array and MERLIN software. We obtained sufficient performance of SNP genotyping in the fingernail samples with the mean SNP call rate of 92.49%, and identified 18 regions with positive LOD scores. Haplotype and linkage analyses with microsatellite markers at these regions confirmed three possible disease-responsible regions, i.e., 5p13.2–q14.1, 15q11.2–q13.1 and 18p11.32–p11.22. Sequence analysis was conducted for ten selected candidate genes at 5p13.2–q14.1, such as *MAP3K1, DAB2, OCLN, FGF10, ESM1, ITGA1, ITGA2, EGFLAM, ERBB2IP,* and *PIK3R1,* but no causative genetic alteration was detected. This is the first experience of adoption of fingernail DNA to genome-wide, high-density SNP microarray analysis, showing candidate brain AVM susceptible regions.

1. Introduction

Arteriovenous malformation (AVM) in the brain is a disease defined by the presence of arteriovenous shunt(s) through a nidus of coiled and tortuous vascular connections between feeding arteries and draining veins within the brain parenchyma [10]. This vascular malformation is thought to be congenital, and develops before or after birth [7] from a residual of the primitive artery–vein connection. Its most common symptom is intracranial hemorrhage with an estimated risk of 1.3–3.9% yearly after the diagnosis of AVM [4]. Other signs may include intractable seizures, headache and ischemic steal syndrome. The prevalence of AVM is estimated to be approximately 0.01% and the detection rate ranges between 1.12 and 1.34 per 100,000 person years [7,10]. Although most cases of AVM are sporadic, a total of 53 patients from 25 families have been reported [27]. Familial brain AVM is defined when it occurs in two or more relatives (up to third-degree relative) in a family without associated disorders such as hereditary hemorrhagic telangiectasia (HHT), is autosomal dominant multisystemic vascular dysplasia [9,27]. It is plausible that familial cases are more frequent and could be overlooked because of asymptomatic conditions in other relatives.

Although several causative genes have been elucidated in some heritable syndromic AVM [2,3,5,6,12,13,17,20,21,23,26], molecular genetic studies of familial or sporadic AVM remain scant. HHT type 1 (HHT1) and HHT type 2 (HHT2) are known to be caused by mutations in *ENG* at 9q34.11 and *ACVRL1* (or *ALK1*) at 12q13.13, respectively [12,17]. Mutations in *RASA1* at 5q14.3 cause capillary malformation-arteriovenous malformation (CM-AVM) [3,6,20,21,26] characterized by small, round-to-oval, pink-red and multiple CM: one-third of CV-AVM patients also has fast-flow lesions such as AVM. Mutations in *PTEN* have been implicated in PTEN hamartoma tumor syndromes including Bannayan–Riley–Ruvalcaba syndrome, in which AVM

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occasionally presents [23]. Three genes, *KIRIT1* (*CCM1*) [13] at 7q21.2, *MGC4607* (*CCM2*) [5] at 7p13 and *PDCD10* (*CCM3*) [2] at 3q26.1, are responsible for cerebral cavernous malformation (hamartomatous vascular malformations). On the other hand, regarding familial AVM, only two linkage analyses using 6 small families have been published by a research group [11,25], showing seven possible disease-responsible regions, i.e., 6q25 with the highest LOD score, 3p27, 4q34, 7p21, 13q32–q33, 16p13–q12 and 20q11–q13, but failed to identify the causative mutation. In sporadic brain AVM, microarray study showed that the *VEGFA*, *ITGA5*, *ENG* and *MMP9* genes that may involve vascular development or maintenance, are highly expressed in AVM compared with normal brain parenchyma [8,22,24].

Here we report results of a genome-wide linkage analysis on an AVM family with four affected members in two successive generations.

2. Materials and methods

2.1. Subjects

A Japanese family consisting of 19 members across four generations included two patients with brain AVM, one patient with pulmonary AVM and one patient with both brain and pulmonary AVM (Fig. 1). The proband (III-3) first exhibited intractable epilepsy at 13 years old and was diagnosed by magnetic resonance imaging (MRI) as having a brain AVM of 2 cm in diameter located in the right frontal lobe (Fig. 2). Chest X-ray at the first visit detected a nodular shadow in the right lower lung field, and a diagnoses of pulmonary AVM with a 24% of shunt-rate was made following angiogram made (Fig. 2). This was resected when the proband was 14 years old. The proband's brain AVM was treated by gamma knife surgery when she was 19 years old, followed by treatment with antiepileptic medication. Her mother (II-3) died of intracranial hemorrhage due to brain AVM, and the maternal grandfather (I-1) died of a cancer. Another patient (III-5) had asymptomatic brain AVM, which was accidentally diagnosed by MRI. His father (II-5) had pulmonary AVM instead of brain AVM. These four members were assigned to "affected", six members (II-6, III-1, III-6, III-7, IV-1, and IV-2) without AVM confirmed by MRI were "unaffected", and the remaining three (I-2, II-1, and IV-3) who were not assessed by MRI but had neither past history of recurrent epistaxis or gastrointestinal tract bleedings were "unknown". None of the members had any AVM-related diseases, such as HHT. Evaluation of cutaneous

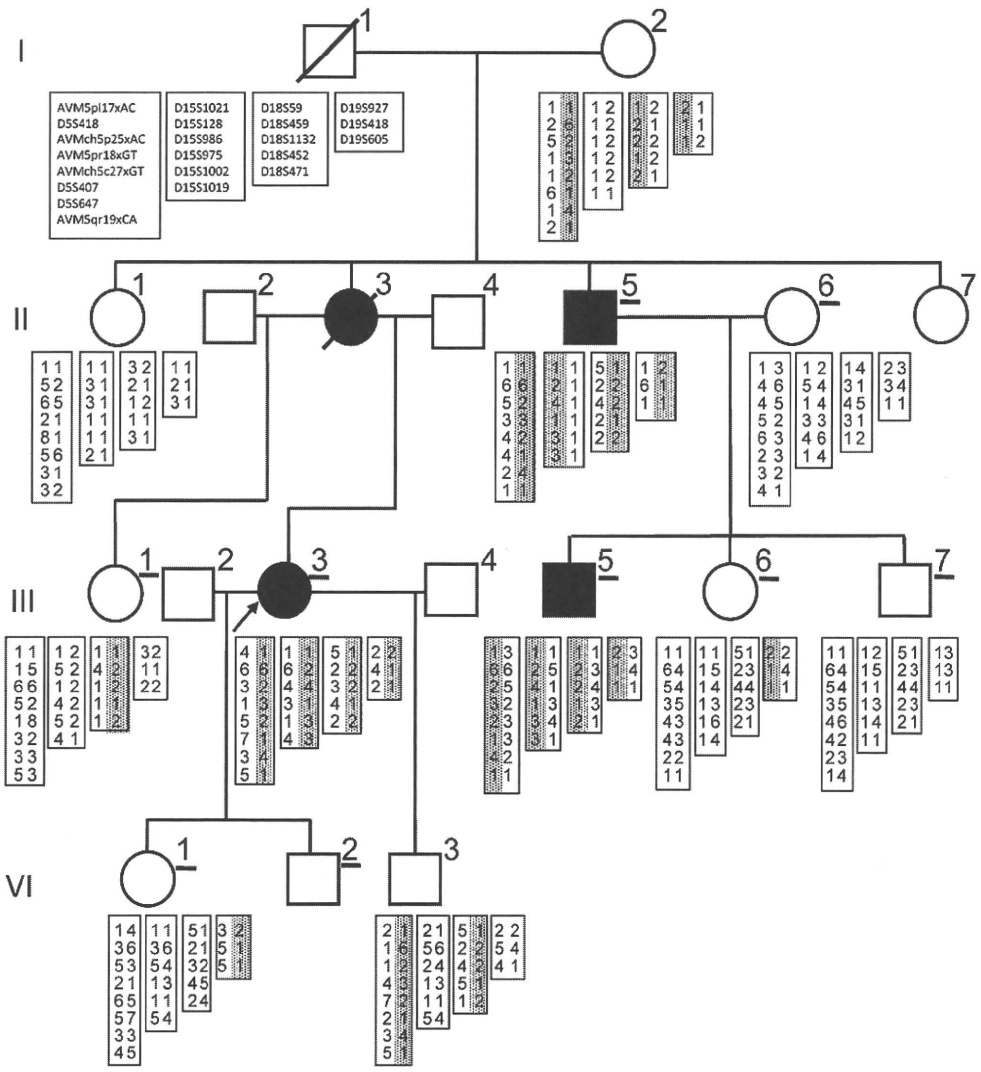

Fig. 1. Results of haplotype analysis at polymorphic loci in four regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42. Underlined individuals indicate those examined by MRI, and DNA was unavailable from individuals without haplotypes. Polymorphic alleles are numbered and candidate disease-associated haplotypes are shown by dotted boxes. Primer sequences designed for CA repeat amplification are available in Supplementary Table.

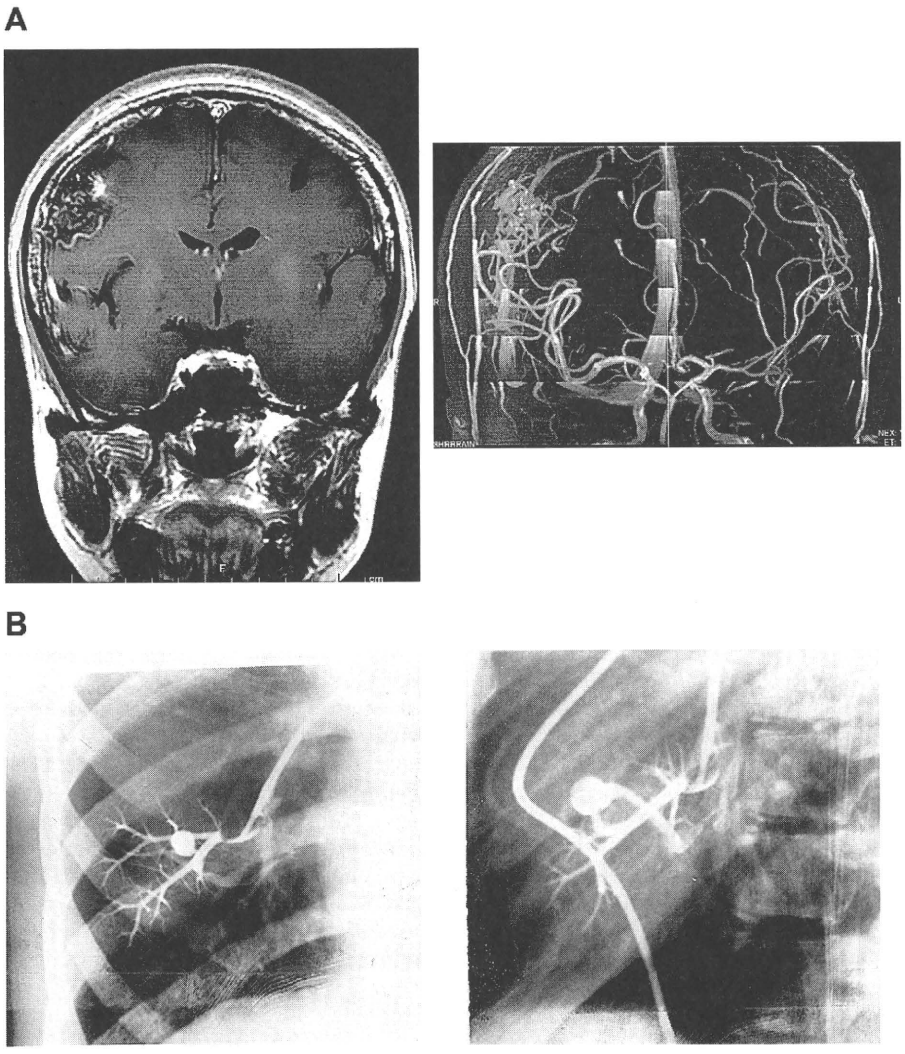

Fig. 2. Imaging of the brain and pulmonary AVM in the proband. (A) MRI scan and MR angiogram of the proband. The AVM is located right frontal lobe measured 2.0 × 1.3 cm. (B) Pulmonary angiograms of the proband. The pulmonary AVM is located in the right lower lobe (rtS8b) with 24% of shunt-rate.

lesions was conducted by examination of the proband and by detailed interview of the other family members by the proband and her sister (III-1), who is nurse. A total of 13 members participated in this study under informed consent. All experimental procedures for this study were approved by Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

2.2. DNA extraction

As a blood sample was available only from the proband, clipped fingernail samples were obtained from 10 of the other 12 members instead. Genomic DNA was extracted from the fingernails using a buffer solution containing urea, DDT and proteinase K, as reported previously [16,18]. Briefly, clipped fingernails were once frozen in liquid nitrogen and crushed into fine powder using Multi-beads Shocker™ (Yasui Kikai, Osaka, Japan). The nail powder was lysed in a urea-lysis solution (2 M urea; 0.5% SDS; 10 mM Tris–HCl, pH 7.5; 0.1 M EDTA) containing 1 mg/ml proteinase K and 40 mM DDT at 55 °C overnight. Nail DNA was extracted with phenol/chloroform, and precipitated with ethanol and sodium acetate. Precipitated nail DNA was dissolved again in extraction buffer (0.5% SDS; 10 mM Tris–HCl, pH 7.5; 0.1 M EDTA) containing 1 mg/ml proteinase K, and incubated at 55 °C overnight. DNA was purified again as above, and was suspended in 30 μl of 1× TE buffer.

2.3. SNP genotyping with Affymetrix 10K 2.0 array

Blood DNA (250 ng) was processed according to the standard protocol provided by the GeneChip Mapping 10K Xba Assay Kit (Affymetrix, Santa Clara, CA). Fingernail DNA was processed in a similar manner but with the two following modifications to adapt to the oligonucleotide microarray system [15]. Prolongation of digestion time from 120 min as the standard protocol to overnight; and increase of the PCR cycle number from 35 to 45 cycles. Data acquired from the Affymetrix GeneChip Operating System were analyzed using the Affymetrix GeneChip Genotyping Analysis Software (GTYPE) 4.0 to call genotypes.

2.4. Linkage analysis with SNP-genotype data and haplotype analysis with microsatellite markers

Multipoint LOD scores were calculated using MERLIN software [1], under an assumption that AVM in the family is transmitted in an autosomal dominant mode with reduced penetrance ($p = 0.9$)

and with the disease allele frequency of 0.001. At loci with a positive LOD score by the GeneChip genotyping, possibly disease-associated haplotypes were constructed using SNP calls.

When SNP information was not informative, microsatellite markers were used for genotyping. Microsatellite markers used were referred to the National Center for Biotechnology Information (NCBI) database. One each of primer pairs for the markers was labeled with FAM, HEX, or NED (Supplementary Table 1), and PCR was performed in a 10 μl mixture containing 5 ng genomic DNA; 0.25 U ExTaq DNA polymerase HS-version (TAKARA Bio Inc., Kyoto, Japan); 200 μM dNTP; 0.5 μM primer; 1× ExTaq buffer on the T1 Thermocycler (Biometra, Goettingen, Germany). PCR products were separated on Genetic Analyzer 3130xl (AppliedBiosystems), and genotyping was carried out using GeneMapper software (AppliedBiosystems). At the regions where the affected individuals have a disease-associated haplotype, two-point LOD score was calculated by MLINK program (included in FASTLINK software version 4.0P) [14].

2.5. Mutation analysis

Some genes located within candidate regions identified by the linkage analysis were selected for further mutation analysis. A few other genes, albeit outside the regions, were also subjected to mutation analysis. Primer pairs for such genes were designed using Primer3-web 0.3.0 (http://frodo.wi.mit.edu/primer3/input.htm), according to their genomic sequences retrieved from the University of California, Santa Cruz (UCSC) Genome Browser Home (http://genome.cse.ucsc.edu/). PCR was carried out in a 15 μl reaction mixture containing 5 ng DNA; 0.25 U ExTaq DNA polymerase HS version; 200 mM dNTP; 0.5 μM each primer; 1× ExTaq buffer on the T1 Thermocycler. PCR products were subjected to direct sequencing, using BigDye Terminator v3.1 Cycle sequencing Kit (AppliedBiosystems) and Genetic Analyzer 3130xl. Electropherograms of sequences were aligned with ATGC software (GENETYX Corp., Tokyo, Japan) to inspect base alterations.

2.6. Search for genomic aberration

To search for copy number change within the candidate loci identified by linkage analysis, we used Affymetrix® Genome-Wide Human SNP Array 5.0 (920,568 probes; Affymetrix). Genomic DNA extracted from white blood cell of proband was processed according to manufacture's protocol. Intensity data from each probes were obtained from Affymetrix® Genotyping Console 3.0 as a CEL files. Unpaired copy number analysis of whole genome was carried out using Partek Genomics Suite (Partek, MO, USA) and regions with copy number change were determined by Hidden Markov Model at default settings.

3. Results

3.1. Linkage and haplotype analyses

The mean SNP call rate was 92.49% in 11 fingernail DNA samples, compared to 98.11% in a blood DNA sample from the proband. Incorrect SNP calls may result in seemingly inconsistent parent–child transmissions, but the call rates obtained are actually enough for further studies. We thus advanced to calculate LOD scores using these data.

The linkage analysis using MERLIN software revealed 18 regions with positive LOD scores (>0.00). Of the 18 regions, 14 with the following conditions were excluded: those without any functional full-length RefSeq genes; those in small size (<200 kb); and those in which some affected members did not have a common haplotype. Consequently, four loci, 5p13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.33–q13.42, remained as possibly linked regions (Figs. 1 and 3).

We then genotyped with microsatellite markers and calculated two-point LOD scores, considering the affected, unaffected, and the unknown family members. We confirmed three of the four candidate loci. They were a 48-Mb region between markers rs1366265 and rs1373965 at 5p13.2–q14.1, a 6-Mb region between rs850819

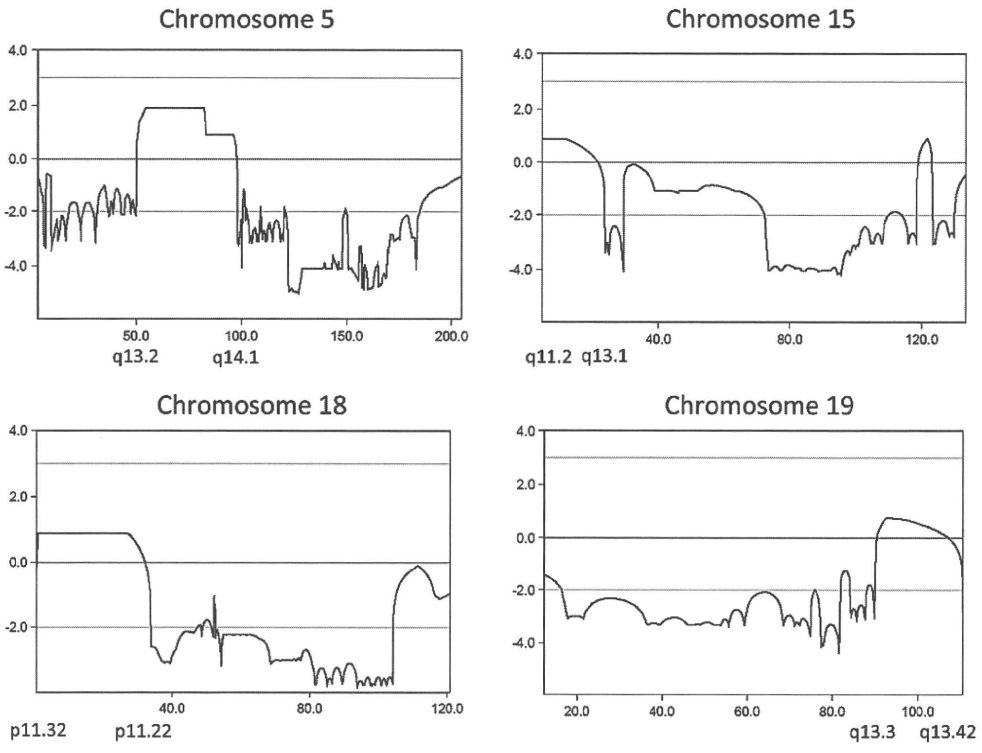

Fig. 3. Multipoint LOD scores calculated by MERLIN in four chromosomal regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42.

and rs818089 at 15q11.2–q13.1, both giving the maximum two-point LOD score of 1.632 ($\theta = 0$), and a 9-Mb region between rs486633 and rs1942150 at 18p11.32–p11.22 with the maximum LOD score of 0.851 ($\theta = 0$) (Table 1). As a possibly disease-associated haplotype on 19q13.33–q13.42 was transmitted to two definitively unaffected individuals (III-6 and IV-1), chromosome 19 was ruled out from the candidacy (Table 1, Fig. 1).

3.2. Mutation analysis of candidate genes

Within the 48-Mb region at 5p13.2–q14.1, there are about 200 RefSeq genes. Ten (*MAP3K1*, *DAB2*, *OCLN*, *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EDFLAM*, *ERBB2IP*, and *PIK3R1*) from these genes were focused and selected as candidates for brain AVM, since they concern development or maintenance of vessels, are associated with other heritable vascular disorders such as HHT, or are expressed in the brain with AVM [8,22,24]. Mutation analyses in these 10 genes revealed no pathologic mutation in the proband, although other affected members were not examined because of insufficient amount of their DNA. Although the genes endoglin isoform 1 precursor (*ENG*), activin A receptor type II like 1 (*ALK1*) and RAS p21 protein activator 1 (*RASA1*) are not located in the candidate region, we investigated whether any of them are involved in the etiology of AVM in the family as a partial symptom of HHT or AVM-CM. Direct sequencing of these three genes failed to show any causative variants.

Copy number analysis of proband revealed one increased copy number loci at 12q and decreased at 2p, 3q, 4q, 6p, 7q and 22q (data not shown). But all these alterations were reported previously as copy number polymorphisms (http://projects.tcag.ca/variation/) and out of our candidate loci. In addition, neither deletions nor microdeletions were detected at 9q34.11 of *ENG*, 12q13.13 of *ALK1* and 5q14.3 of *RASA1*.

4. Discussion

We have reported a family consisting of two affected members with brain AVM, one with pulmonary AVM and one with both brain and pulmonary AVM. The condition in this family met the criteria of familial brain AVM and seems to be inherited in an autosomal dominant mode. We tried to assign the location of a putative disease-gene by linkage analysis and search for mutations by subsequent candidate gene approach.

The linkage analysis of the family revealed three candidate regions (5p13.2–q14.1, 15q11.2–q13.1, and 18p11.32–p11.22) with relatively high LOD scores of 1.632, 1.632 and 0.851, respectively (Table 1). However, neither region was conclusive. This insufficient mapping may have arisen from the small pedigree size, and/or from incomplete ascertainment of affected members, e.g., probable existence of asymptomatic affected persons among the "unknown" members. Indeed, as for a candidate locus at 5p13.2–q14.1, the proband's maternal grandmother (I-2) and son (IV-3) had a haplotype common to the three affected members (Fig. 1), but they were fallen into the "unknown" individuals. If DNA from IV-2 was available and if MRI examinations of VI-3 and I-2 were carried out, we would have obtained more definitive results. As we performed linkage analysis using high-density SNP genotyping, 14 small regions not containing RefSeq genes or miRNAs showed a positive LOD score. It is possible that an unidentified transcribed RNA in one of these regions could cause familial AVM, but these regions are candidate loci with a lower priority than those containing known genes. Thus, the three regions have remained at present as the equally possible loci for AVM. The three regions do not overlap with a previously reported candidate locus of familial brain AVM, i.e., 6p25 [11], and do not contain genes responsible for syndromic AVM (heritable disorders involving AVM) or cerebral cavernous malformations, such as *ENG* [17], *ALK1* [12], *RASA1* [3,6,20,21,26], and *PTEN* [23], *KRIT1* [13], *MGC407* [5], *PDCD10* [2].

We then searched for mutations in 10 genes within 5p13.2–q14.1, among which *MAP3K1*, *DAB2* and *OCLN* encode proteins playing roles in the TGF-β signaling pathway, and *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EGFLAM*, *ERBB2IP* and *PIK3R1* were those expressed in brain AVM tissues by previous microarray analysis [8,22,24]. Nevertheless, no pathologic mutation was found in any of them. Because the presence of both brain AVM and pulmonary AVM in this pedigree is reminiscent of Hereditary Hemorrhagic Telangiectasia, we analyzed *ENG* and *ALK1* for mutations and genomic aberrations, which may cause HHT1 and HHT2 respectively [12,17]. The proband did not have any mutations in the coding exons or intron/exon boundaries of either gene, nor any genomic aberrations at those loci. We also analyzed *RASA1* because this may cause CM-AVM, which is characterized by multiple CM and AVM [3,6,20,21,26]. No causative mutation or genomic aberration was detected in the proband. Although other genes, such as *KRIT1*, *MGC407* and *PDCD10*, have been shown to cause slow-flow lesions i.e., cerebral cavernous malformation [2,5,13], they were not investigated in the present study, because the clinical manifestations in our family did not meet the criteria for these diseases.

Participation of family members and compliance with guidelines for human genome researches are critical to conduct a linkage analysis. Whole-blood samples cannot occasionally be available in some family members because of their far domicile. In such the case, fingernail DNA is useful, since clipped fingernails can be mailed in a usual way, and stored long at a room temperature, as indicated previously [16,19]. The present study is the first experience to adopt fingernail DNA to genome-wide high-density SNP microarray analysis. The performance obtained from fingernail DNA was sufficient, showing all SNP call rates of >86%. According to the manufacture's protocol, samples with an SNP call rate of <85% should further be evaluated before including the data in downstream analysis. Incorrect SNP calls may make serious problems in linkage analysis. For instance, SNPs with parent–child transmission inconsistency may be omitted, leading to a reduced LOD score.

In conclusion, we have assigned the familial AVM locus to three alternative regions, 5p13.2–q13.2, 15q11.2–q13.1 and 18p11.32–p11.22, by a genome-wide, high-density, SNP-based

Table 1
Two-point LOD scores for brain AVM at various loci.

Locus	Recombination fraction (θ)					
	0.00	0.01	0.02	0.03	0.04	0.05
AVM5pl17xAC	0.032	0.030	0.029	0.027	0.026	0.025
D5S418	0.551	0.535	0.518	0.501	0.484	0.467
AVMch5p25xAC	1.334	1.301	1.268	1.234	1.201	1.167
AVM5pr18xGT	0.511	0.491	0.472	0.452	0.433	0.414
AVMch5c27xGT	1.630	1.597	1.564	1.531	1.497	1.463
AVM5c18xAC	1.373	1.344	1.314	1.285	1.255	1.225
D5S407	1.632	1.599	1.566	1.532	1.499	1.465
D5S647	1.154	1.121	1.089	1.056	1.023	0.991
AVM5qr19xCA	0.810	0.790	0.769	0.748	0.727	0.706
D15S1021	0.171	0.164	0.157	0.150	0.143	0.137
D15S128	0.876	0.858	0.841	0.823	0.805	0.787
D15S986	0.812	0.791	0.770	0.749	0.728	0.707
D15S975	0.400	0.387	0.374	0.361	0.348	0.335
D15S1002	1.330	1.298	1.266	1.234	1.202	1.170
D15S1019	1.632	1.599	1.566	1.532	1.499	1.465
D18S59	0.199	0.214	0.225	0.234	0.241	0.246
D18S459	0.142	0.136	0.131	0.125	0.120	0.114
D18S1132	0.677	0.663	0.650	0.636	0.623	0.609
D18S452	0.851	0.832	0.813	0.794	0.774	0.755
D18S471	0.240	0.231	0.222	0.214	0.205	0.197
D19S927	−0.302	−0.277	−0.254	−0.234	−0.216	−0.200
D19S418	−2.655	−2.453	−2.257	−2.078	−1.919	−1.778
D19S605	−0.648	−0.574	−0.512	−0.460	−0.414	−0.374

linkage analysis with fingernail DNA in an AVM family. However, mutation analyses of some genes in the regions failed to identify any pathological changes.

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Competing interests

There are no competing interests.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmg.2010.06.007.

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RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Ruvalcaba Syndrome Revisited

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TO THE EDITOR:

Ruvalcaba et al. [1971] reported a family with a previously undescribed malformation syndrome inherited as an autosomal dominant trait, which was subsequently termed Ruvalcaba syndrome. The hallmarks were described to be short stature; microcephaly with developmental delay; characteristic facies; and skeletal anomalies, including short metacarpals and osteochondritis of the spine. Since then, however, Ruvalcaba syndrome has not gained a solid consensus as a distinctive disorder because of the small number of additional reports and its phenotypic overlap with other malformation syndromes. Here, we report a girl whose manifestations were identical to those of the original patients. We believe that the syndromic constellations of the present girl and previously reported patients are sufficient to regard Ruvalcaba syndrome as a separate disease entity.

The proposita was the first child born to healthy, non-consanguineous, Japanese parents, a G1P0 27-year-old woman and a 34-year-old man. The mother had undergone acetabuloplasty for right-sided acetabular dysplasia at age 20 years, and she showed downslanting palpebral fissures. The family history was otherwise unremarkable. The child was vaginally delivered at term with cephalic presentation. The delivery was preceded by premature rupture of the amniotic membrane, but no perinatal asphyxia was evident. The child's birth weight, birth length, and OFC were 1,868 g (−3.6 SD), 42 cm (−4.8 SD), and 28.5 cm (−3.6 SD), respectively. She was referred to our hospital at age 6 months because of her failure to thrive, multiple congenital abnormalities, and developmental delay. A physical examination showed microcephaly (−4.7 SD) with brachycephaly, small and downslanting palpebral fissures, a beaked nose with hypoplastic nasal alae, synophrys, and small hands and feet. Orthopedic assessment identified bilateral talipes planus together with talipes valgus, pectus excavatum, scoliosis, and restricted motion of the hip joints. The patient's karyotype was 46,XX, and her motor development was mildly retarded: she was able to keep her head steady at 5 months, she could sit without support at 11 months, and she was able to walk alone at 32 months. Other medical problems included dysopia with myopia, a convulsive episode at age 7 years, and surgery for acute gastric volvulus at age 8 years. Hearing impairment developed following repetitive otitis media and eventually necessitated hearing aids. A skeletal survey performed at age 9 years (Fig. 1) showed short IV and V metacarpals and an irregular spinal endplate. She is currently 13-year old (Fig. 2). She presents with subtle breast development but without pubic hair. She is very short (121 cm, −5.9 SD) and is mentally retarded (IQ <35). With the informed consent of her guardians, she underwent molecular analysis for *TRPS1* and *GNAS1* as well as 244K-CGH microarray analysis (Agilent Technologies, Santa Clara, CA). The results were all negative.

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To date, only seven patients with Ruvalcava syndrome have been reported, and we consider the present girl to be the eighth case. The clinical features of the present girl and those of the other patients who were reported to have Ruvalcaba syndrome are summarized in Table I. Ruvalcaba et al. [1971] originally reported two brothers and two "partially affected" female relatives who were maternal cousins of the index brothers. However, there have been too few subsequent reports on Ruvalcaba syndrome to attract general attention to the disorder. In addition, it was unfortunate that other malformation syndromes have been mistakenly reported as Ruvalcaba syndrome. A Japanese family reported by Sugio and Kajii [1984] was initially described as Ruvalcaba syndrome; however, the diagnosis was later revised to tricho-rhino-phalangeal syndrome (TRP) type III, which

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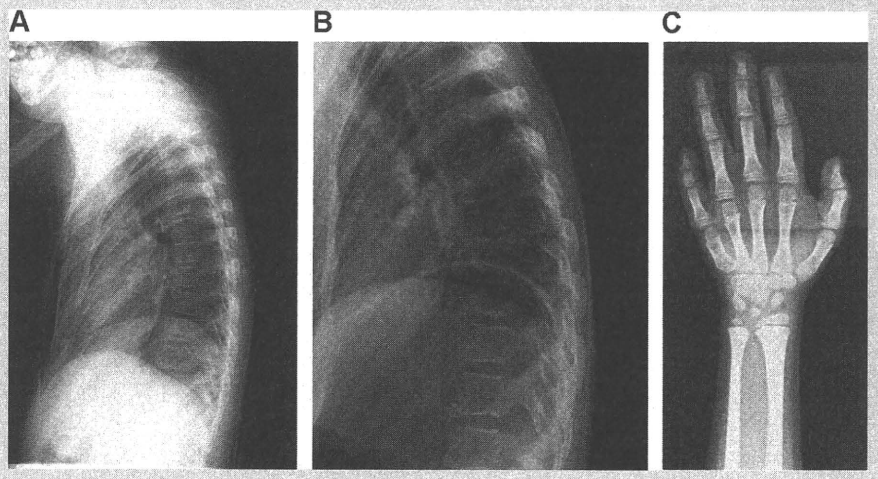

FIG. 1. Skeletal radiographs of the patient. A,B: Lateral views of the spine taken at age 9 and 12 years, respectively. Note the irregular endplate of the spine. C: Hand and wrist at age 9 years showing shortening of the IV and V metacarpals.

is now known to be caused by *TRPS1* mutations [Lüdecke et al., 2001]. The original patients with Hunter–McAlpine syndrome (HMS) were also misreported as having Ruvalcaba syndrome [Hunter et al., 1977]. Currently, this craniosynostosis syndrome is considered to result from a duplication of 5q35-qter. These diagnostic issues have reduced the consensus around Ruvalcaba syndrome.

Ruvalcaba syndrome is apparently distinctive from TRP type III and HMS. TRP type III is distinguishable from Ruvalcaba syndrome based on the absence of mental retardation and microcephaly and the presence of sparse hair in TRP type III, as is HMS on the basis of very mild shortening of the metacarpals and the presence of craniosynostosis. Facial dysmorphology is also different among Ruvalcaba syndrome, TRP type III, and HMS. In particular, downslanting palpebral fissures appear to be characteristic of Ruvalcaba syndrome (Table I). We were not able to find a *TRPS1* mutation or submicroscopic chromosomal rearrangement in the present girl.

As mentioned in the original report [Ruvalcaba et al., 1971], the brachydactyly found in Ruvalcaba syndrome superficially resembles that of Albright hereditary osteodystrophy. As solidified by the absence of a *GNAS1* mutation in the present girl, Ruvalcaba syndrome is apparently distinct from pseudohypoparathyroidism and pseudopseudohypoparathyroidism. In terms of syndrome nosology, Ruvalcaba syndrome should not be confused with Bannayan–Riley–Ruvalcaba syndrome, which refers to a hamartomatous syndrome caused by a *PTEN* gene mutation [Lachlan et al., 2007].

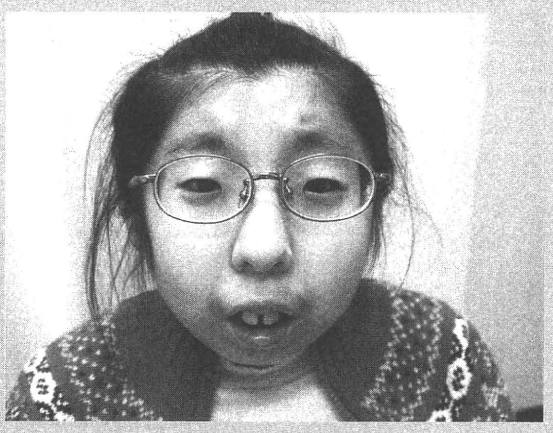

FIG. 2. Facial features of the patient at age 12 years, showing a beaked nose, downslanting palpebral fissures, and microstomia with crowded teeth. Synophrys was absent at this age.

TABLE I. Clinical Manifestations of Patients With Ruvalcaba Syndrome, Coupled with Those in Related Syndromes

	Ruvalcaba et al. [1971]				Georgmaneanu et al. [1978] (M)	Bianchi et al. [1984] (M)	Bialer et al. [1989] (F)	This report (F)	Tricho-rhino-phalangeal syndrome type III	Hunter–McAlpine syndrome
	1 (M)	2 (M)	3 (F)	4 (F)						
General										
Developmental delay	+	+	+	+	+	+	+	+		+
Short stature	+	+	+	+		+	+	+	+	+
Delayed adolescence	+	+		+		+	+	+		+
Craniofacial										
Microcephaly	+	+			+	+	+	+		+ (craniosynostosis)
Downslanting palpebral fissures	+	+		+	+	+		+		
Abnormal nose	+	+		+	+	+	+	+	+	+
Narrow maxilla	+	+	+	+	+	+	+	+	+	
Microstomia	+	+	+	+	+		+	+	+	+
Skeletal										
Narrow trunk	+	+			+	+		+	+	
Pectus deformity	+ (C)	+ (C)	+ (C)		+ (C)		+ (E)	+ (E)	+	
Scoliosis/kyphosis	+	+	+	+	+	+	+	+	+	
Osteochondritis of spine	+	+		+				+	+	
Limitation of joint extension	+	+			+	+		+		+
Short limbs	+	+	+	+		+			+	
Small hands	+	+		+		+	+	+	+	+
Small feet	+	+		+		+	+	+	+	
Short metacarpals	+	+		+		+	+	+	+	
Incurved 5th fingers	+	+	+	+	+				+	+
Short phalanges	+	+		+		+	+	+	+	+
Urogenital										
Undescended testes	+	+			+					
Hypoplastic genitals	+	+		+	+					
Renal abnormality	+	+			+		+			
Others										
Seizure								+		
Inguinal hernia	+	+								
Gastroenteral involvement							+	+		
Ocular involvement						+		+		
Hearing impairment								+		

M, male; F, female; +, present; blank space, absent or not mentioned; C, carinatum; E, excavatum.

Ruvalcaba et al. [1971] suggested that Ruvalcaba syndrome shows an X-linked semi-dominant inheritance pattern, based on the mild manifestation of affected females; yet this can be ruled out by the complete manifestation of the affected girl described here. The mother of the present girl had acetabular dysplasia as well as minor dysmorphic features (downslanting palpebral fissures). Bianchi et al. [1984] stated briefly that the affected boy and his mother shared some facial characteristics. In addition, Georma-neanu et al. [1978] also reported that the father of the proposita had clinodactyly. Thus, it is tempting to assume that Ruvalcaba syndrome is inherited as an autosomal dominant trait with variable penetrance, and that the symptoms in patients with mild manifestation are quite variable.

The etiology of Ruvalcaba syndrome remains unknown. The proposita reported by Georgmaneanu et al. [1978] was associated with a t(13q;14q) balanced translocation. However, this finding was not consistent with those of other affected individuals. At present, the diagnosis of Ruvalcaba syndrome entirely depends on clinical findings including skeletal radiology. Accordingly, suitable diagnostic criteria should be developed using the findings from definitive cases. The diagnostic keys include a very short stature; microcephaly; developmental delay; characteristic brachydactyly; and facial dysmorphology including downslanting palpebral fissures, a beaked nose, a narrow maxilla, and microstomia. Although spinal osteochondritis is indeed a distinguishing finding in Ruvalcaba syndrome, it was very subtle in the present girl. She was notably associated with gastric volvulus, as was an affected female reported by Bialer et al. [1989] with a hiatal hernia and gastroesophageal reflux. Therefore, affected individuals may require careful monitoring for gastroenteral problems.

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神経線維腫症1型における分子細胞遺伝学的スクリーニング

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要旨　神経線維腫症1型large deletion症例のスクリーニングとしてBACクローンによるFISH解析を導入した。37例の定期医療管中の症例のうち、5例で解析を行い、欠失症例は認めなかった。NF1 large deletion症例は、過成長、顔貌異常、より早期からの神経線維腫の出現、学習障害などが特徴と考えられ、比較的容易に行うことができるFISH解析は、本症の医療管理を行う小児専門医療機関では考慮すべき検査と考えられた。

Fluorescence in situ hybridization analysis for detecting a large deletion of *NF1* gene in *NF1* patients.

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Neurofibromatosis type 1 (NF 1) is an autosomal dominant disorder (OMIM. 162200), characterized by cutaneous manifestations including café-au-lait patches and dermal neurofibromas, and multi-organ system disorders. After the identification of NF 1 gene, molecular analysis has been making it possible to understand the pathogenesis of NF 1. The deletion of entire gene of NF 1 is associated with severe phenotype including dysmorphism, mental retardation, and early onset of a large number of neurofibromas. We established the screening system for detecting the large deletion of NF 1 gene region by FISH with probes derived from bacterial artificial chromosome (BAC) clones.

Key words：Fluorescence in situ hybridization (FISH). Neurofibromatosis type 1 (NF 1). Bacterial artificial chromosome (BAC) deletion

はじめに

神経線維腫症1型（Neurofibromatosis type 1, NF1）は、全身の腫瘍性病変と色素斑を特徴とする常染色体優性遺伝病で、原因遺伝子は17q11.2にマップされる*NF1*[1]である。NF1は発生頻度約3000出生に1例で、小児科領域でも比較的経験することが多い疾患である。本症においては根本治療は困難なものの、合併症管理においては年齢ごとの出現しやすい兆候を把握して管理することが重要であり、診断基準や合併症の出現時期や頻度などは、成書でもまとめられている[2]。

*NF1*遺伝子はアミノ酸コードエクソンが58、コードするアミノ酸が2839で、全長が約350kbに及ぶ大きな遺伝子である。変異が起こる割合は他の遺伝子より高く、de novo変異が50%以上を占める理由になっている。多くの変異が遺伝子内欠失や点変異であるが、いわゆるgenotype-phenotypeの相関はあまりないとされている。しかし、*NF1*遺伝子

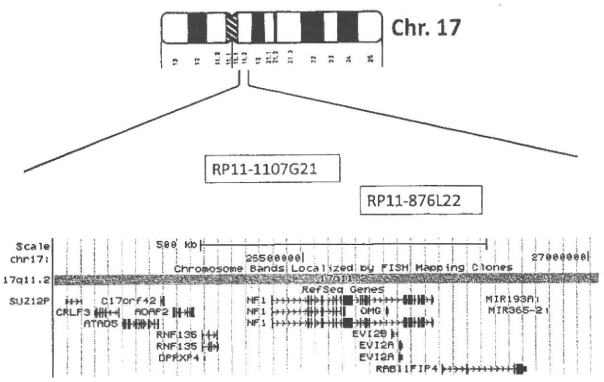

図1．FISH解析で用いたBAC cloneとそのマップ上の位置関係。2つのクローンRP11-1107G21とRP11-876L22とで、NF1領域をカバーする形となっている。

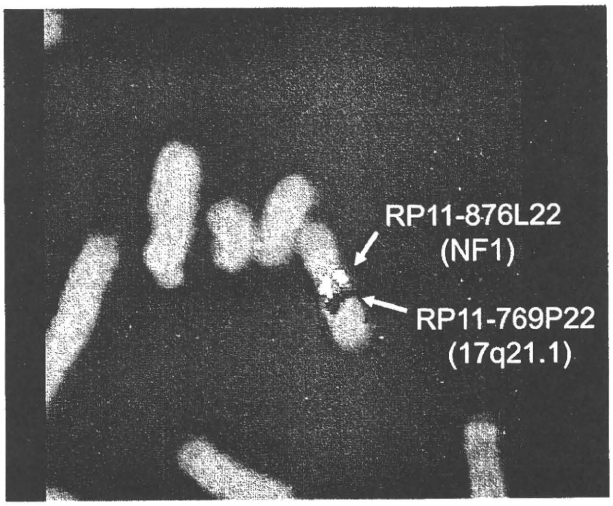

図2．FISH解析での検出されたシグナル。

全体を含む領域の欠失は精神遅滞や顔貌異常などを呈し、一定のgenotype-phenotype相関が認められる[3]。

NF1を含めた先天異常症候群の多くが、小児専門医療機関で医療管理を受ける場合が多い。ここでは、定期医療管理を行ってきた37例の合併症の概要をまとめ、合わせておこなってきたFISHによるNF1欠失スクリーニングについてまとめた。

対象と方法

対象は2001年4月から2009年8月までに、神奈川県立こども医療センター遺伝科を受診したNF137症例で、男女比は18：19でほぼ同じであった。このうち6家系で家族歴を認めていた。観察体制は、3歳以下の乳幼児期は3－6カ月ごとの定期受診、3歳以降学童期には約1年ごとの定期受診体制とした。診断は、NIH Consensus Conference criteria[4]にもとづいた。合併症や身体計測に関する情報は、診療録を参考とした。

NF1を含む全遺伝子欠失のスクリーニングは、bacterial artificial chromosome（BAC）クローンをプローブとしてFISHにて行った。BACクローンは、UCSC Genome Browser 2004にもとづいてNF1遺伝子の1部を含むクローンを複数選択した。17番染色体のコントロールプローブも同じくUCSC Genome Browser 2004に従い選択した。BACクローンは、単クローンを適切な抗生剤を含むLB培地で一晩培養し、Miniprep Spin Colum（キアゲン）を用いてBAC DNAを抽出した。抽出BAC DNAは、Nick translationによりラベリング（Vysis-Abbot）を行った。コントロールプローブとのコントラストを図るために、それぞれSpectrum Green、Spectrum Orange（Vysis-Abbot）でラベリングを行った。観察は、Leica CW4000を用いて、付属CCDカメラで観察した。末梢血液リンパ球は、標準的方法で培養処理を行い、標本作製を行った。解析は、十分な説明の後に保護者の承諾の下で行った。

結果と考察

初診年齢の分布は、0－1歳が14例、2－5歳が12例、6－10歳が8例、11－15歳が2例、16歳以上が1例（遺伝カウンセリングを目的とした30歳女性）で、乳幼児期早期からの紹介受診例が多かった。初診時に明確に認めた骨格異常としては、Anterolateral bowing of the tibiaが3例で認められた。乳幼児期の腫瘍発生では初診時すでに2例で認め、片側上下眼瞼（2歳女児）、眼窩内腫瘍（1歳女児）であった。12例で視神経MRIの画像診断が行われたが、視神経膠腫は認められなかった。身体計測では、ほとんどの症例が幼児期までに相対的な大頭症

を認めた。

BACクローンによるFISHによるNF1欠失スクリーニングの適応は、精神遅滞、過成長が目立つ、顔貌異常など奇形徴候を伴う、早期からneurofibromaが多発する、などの症状を認めた場合があげられる。今回の解析では5症例で解析を行った。選択した検出プローブはそれぞれ、RP11-1107G21とRP11-876L22で、コントロールプローブに17q21.1にマップされるRP11-769P22（17q21.1微細欠失症候群検出プローブ）を用いた（**図1**）。結果は5例いずれも欠失は認めなかった（**図2**）。

大橋らは、*NF1*領域のBAC、RP11-876L22を用いたFISH解析により82症例中6例（7%）にNF1遺伝子欠失を検出した。その臨床特徴は眼間開離、幅広い眉毛、短くて丸い鼻、低い鼻根部、粗な頭髪等が共通した。ほかに心血管奇形奇形の合併を1例で認めたが、明らかな学習障害がみられる症例はなかったとしている[5]。一般に、large deletionはNF1全体の5%程度とされており、その検出法は今回のBACクローンでのFISHやMultiplex ligation-dependent probe amplification（MLPA）法が用いられる。これまでの報告例でも、大橋らが報告した顔貌異常のほかに過成長が特徴とされている[6]。一般に、NF1においてはgenotype-phenotypeの相関は乏しいとされて、同じ変異を共有する同一家系内でも臨床症状は大きく異なることがあるとされている。すべてのNF1症例で、BACクローンによるFISH解析で欠失解析が適応となるわけではないが、過成長や顔貌異常を伴う非典型例においては考慮するべき検査法と思われる。また、欠失例はより早期から神経線維腫が出現しやすいとされるため、医療管理の面からも解析は必要と考えられる。FISH解析はすでに遺伝学的検査としては臨床一般検査となっていることから、欠失スクリーニングを継続する意義はあると考えられた。

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遺伝学的検査

細胞遺伝学的診断のアルゴリズム

Algorithm in cytogenetic diagnosis

黒澤健司

Key words：アレイCGH，染色体，copy number changes，遺伝カウンセリング

はじめに

アレイCGH解析法は，細胞遺伝学的診断に革命をもたらしたが，それによってこれまで行われてきた標準ギムザ-トリプシン分染法(G分染法)がなくなるという話は聞いていない．FISH法も同様である．新しい細胞遺伝学的診断方法は，それぞれ画期的であるが，完全に置き換わることは少なく，従来法を補完する役割を担っている．このことは，核型標準記載法ISCN 2009(Karger)をみてもわかる．しかし，臨床現場に至っては，この使い分けを適切に行わないと大きな誤りを犯すことになる．現在，この問題に関してはもう一度整理し直す時期にきているようである．

本稿では，こうした細胞遺伝学的各種解析方法を整理して，そのアルゴリズムをまとめてみたい．

1. なぜ染色体か？—先天異常病因検索としての染色体検査—

一般集団における先天異常・奇形の発生頻度は約3％に及ぶ．先天異常は新生児・乳児期の死亡原因の約1/3を占め，その対応は医学上大きな課題である．先進国の小児病院では入院患者における先天異常・遺伝性疾患の占める割合は約6割に達している．そのなかでも染色体異常症の占める割合は低くない(**表1**)．また，先天異常を原因別に分類すると，その多くが原因不明ないしは多因子遺伝・環境要因であるが，いずれにしても明確に原因を特定することはできない範疇である．しかし，その次に原因として挙げられるのは染色体異常症であり，染色体検査で原因を明確にできる疾患範疇といえる．つまり，染色体検査は，一般集団の3％に及ぶ先天異常の原因検索として極めて重要な位置にある．新しい遺伝学的解析方法が開発されつつあるが，染色体検査は最も簡便でありながら，最も効率よく先天異常の原因を同定できる手段

表1 小児病院ハイケアユニット病棟における先天異常・遺伝性疾患患者の占める割合(900例の検討)

疾患	割合(%)
単独の先天異常	21.3
染色体異常症	12.7
既知奇形症候群	11.2
その他の単一遺伝病	4.8
ミトコンドリア遺伝病	0.9
代謝疾患	1.2
後天性疾患	42.4

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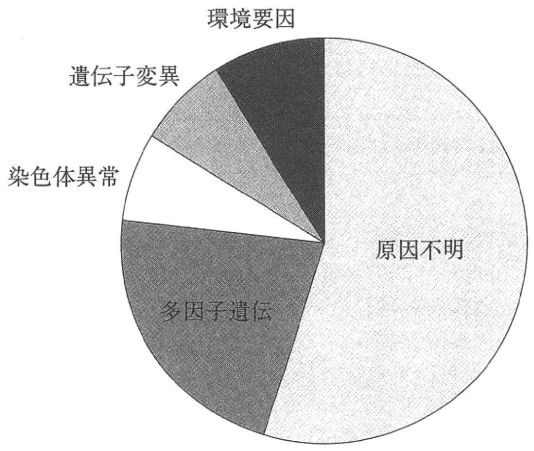

図1 先天異常の原因分類

である(**図1**).

2. 染色体検査でわかること

a. 染色体検査法の選び方—染色体検査の適応と染色体異常—

先天異常の染色体検査の適応としては，転座保因者診断を別にすると精神遅滞や多発形態異常，成長障害など何らかの臨床症状が挙げられる(**表2**)[1]．こうした症状の由来は，量的効果(欠失，重複)，再構成による遺伝子切断がもたらす効果，染色体の親由来による効果(ゲノムインプリンティング)，位置的効果などがある(**表3**)．かつ，染色体異常の結果(効果)のすべてが実際に目に見える'症状'として現れるわけではなく，現れ方には一定のスペクトラムが存在して，極端な場合，受精後着床さえしないことも想定する必要がある(**表4**)[2]．これは，染色体異常全体を氷山に例えると，我々が実際に目にしている染色体異常症例は，その水面上に現れたほんの一部であるともいえる．**表2**からわかるとおり，目的とする疾患や臨床症状により，染色体検査において注目すべき点や追加すべき項目，解析方法も全く異なる．結果として報告書に記載するコメントも異なってくる．検査を行うにあたって臨床情報が極めて重要であることは，強調されすぎることはない．

b. どこまでわかるか？—G分染法の検出限界と追加検査—

末梢血リンパ球でのG分染法の観察可能な解像度は，550バンドレベルでは理論的には約5 Mbまでの染色体再構成を検出できる(高精度分染850バンドレベルでは約3.5 Mb)．これは，遺伝子約50個に相当(同約30個)する．しかし，実際には白バンド同士などの転座の確認は難しく，領域(例えば1番染色体短腕末端部)によっても検出困難な領域は存在する．

染色体の観察は，少なくとも20細胞を観察する('遺伝学的検査としての染色体検査ガイドライン'2006.10.17. 日本人類遺伝学会)が，低頻度モザイクは検出できないことがある．**表5**に観察細胞数と除外できるモザイク率をまとめた[3]．20細胞中1細胞に数的異常が認められたら，更に10あるいは30細胞を観察する．同じ異常が2細胞以上にあれば真のモザイクと判定する．症状から当該染色体異常が疑われるが，50細胞まで増やしても1細胞にしか異常を認めない場合には，他の組織(培養皮膚線維芽細胞の染色体分析や頬粘膜細胞間期核FISH)で検討する．9番染色体トリソミーモザイクは，数%の低頻度モザイクであることが少なくなく，臨床症状からある程度予想をつけて解析を進めないと見落とす可能性がある．同じモザイクでも疾患によっては組織特異的モザイクを検出する必要がある．その代表がPallister-Killian症候群で，皮膚線維芽細胞の染色体分析でi(12p)のモザイクを確認する．12番染色体動原体αサテライトプローブでの頬粘膜細胞間期核FISHは，非侵襲的検査として有用である．

過剰マーカー染色体(extra structurally abnormal chromosome: ESAC，あるいは，supernumerary marker chromosome: SMC)は由来を通常のGTGで同定できない微小なマーカー染色体で，多くが*de novo*で発生している．しかし，親が過剰マーカー染色体をもつときは，モザイクの有無と症状を慎重に検討する必要がでてくる．また，親が均衡型相互転座保因者で減数分裂の際に3：1分離により過剰マーカー染色体が生じる場合があることも注意する(例：t(11;

表2　染色体検査の適応と目的（文献[1]より改変）

適応	主な検査	追加検査	主な疾患
既知の数的異常	GTG		13 trisomy，18 trisomy，Down 症候群
発達遅滞，成長障害，多発奇形	GTG	FISH，MLPA，アレイ CGH	不均衡型転座，微細欠失
既知の奇形症候群	FISH		Williams 症候群，22q11.2 欠失症候群
		メチル化解析	Prader–Willi 症候群/Angelman 症候群
精神遅滞男性		PCR サザン	脆弱 X 症候群
低身長女児	GTG		Turner 症候群
二次性徴遅延，不妊	GTG	FISH	Klinefelter 症候群
免疫不全，日光過敏	GTG	SCE 観察	Fanconi 貧血，Bloom 症候群など染色体断裂症候群
習慣性流産，不育症	GTG		転座
染色体異常児の親	GTG		転座

表3　染色体異常が形質の変化（症状）をもたらすメカニズム

1. 量的効果（dosage effects）
   例：モノソミー，トリソミー，欠失，重複
2. 染色体再構成による遺伝子切断
   例：均衡型相互転座により Mendel 遺伝病の発症
3. ゲノムインプリンティング
   例：Prader–Willi 症候群と Angelman 症候群
4. 染色体再構成による位置的効果

22））．GTG 検査としての限界の一つであるが，ある程度同定可能なこともある．特にサテライトを有する場合には FISH プローブと臨床像の組み合わせから対応できることがある．

出生前に診断された *de novo* の均衡型転座のうち，実際に異常所見（症状）を呈するものは 6.1％程度といわれている[4]．均衡型転座で症状がでるメカニズムとしては，①量依存性の遺伝子の転座切断による遺伝子発現の低下，②*cis* の位置関係にある発現調節領域の転座がもたらす分離破壊による特定遺伝子の発現低下，③転座による片親ダイソミーがインプリンティングを受ける遺伝子発現の不均衡をきたす，などが考えられてきた．そして一般的には①の場合がほとんどであろうと推測されてきた．ごく最近になり，症状をきたす均衡型転座，特に数カ所で切断をきたしながら均衡型を保つ例を高精度アレイ CGH で解析すると，実際には切断点において微細な欠失や重複が比較的高い頻度で生じていて，変異由来では精子形成由来が多いことがわかってきた[5]．それどころか，症状のない均衡型相互転座保因者の中にも切断点での微細欠失が多く検出されることがわかった．しかも，こうした切断点領域には明確な特徴があまり認められていない．ゲノム病といわれる微細欠失・重複症候群の発症メカニズムにかかわる繰り返し配列（low copy repeats: LCRs, あるいは segmental duplication）とは無関係に転座が起こっている．こうした事実は，遺伝カウンセリング上極めて重要である．1つは，*de novo* の均衡型転座が羊水検査で判明した場合の予後判定であり，もう1つは両親のいずれかが均衡型転座と判明している場合に児が両親と同じ転座を有しているからといって本当に両親と同じく症状のない保因者となりえるかという問題である．アレイ CGH の臨床応用が一つの手がかりになる可能性がある．

3. FISH 検査でわかること

FISH 検査を進めるうえで考慮すべき点として，①疾患ごとに考えた場合，FISH で診断可能な微細構造異常例はその疾患全体のどれくらいの割合を占めているのか？，②FISH により診断可能な疾患では，FISH 検査で臨床症状をどこまで推測できるか（FISH 解析による genotype–phenotype 相関をどこまでいえるか）？，③FISH 検査の解像度はどれくらいか（診断可