Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai et al., 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi et al., 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo et al., 1997; Smith et al., 2003; Kobayashi et al., 2006, 2009; Wood et al., 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. SI). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (LINE1) and Alu repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi et al., 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence in situ hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed H19 and KCNQ1OT1 (LIT1) gDMRs, for SRS we assayed the H19 gDMR and for PWS and AS we assayed the SNRPN gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of H19 at chromosome 11 is known to be a frequent occurrence in SRS (Bliek et al., 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV), and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1O-T1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al.*, 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite

low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al.*, 2007), *in vitro* culturing (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003), cryopreservation (Emiliani *et al.*, 2000; Honda *et al.*, 2001) and the timing of embryo transfer (Shimizu *et al.*, 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al.*, 2004; Kobayashi *et al.*, 2007; Marques *et al.*, 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS-2	IVF-ET	H19 hypomethylated (mosaic)			
SRS-3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS-4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS-5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS-6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS-8		H19 hypomethylated			
SRS-9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS-14		H19 hypomethylated			
SRS-15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson et al., 2005; Bliek et al., 2006; Kagami et al., 2007; Galli-Tsinopoulou et al., 2008). Our population-wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim et al. (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal

methylation at additional imprinted loci. However, while Bliek et al. (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of KCNQ1OT1(LIT1) ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol et al. (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes in vitro. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large-scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative

methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

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Conflict of interest

None declared.

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BRIEF COMMUNICATIONS

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genetics

Mutations affecting components of the SWI/SNF complex cause Coffin-Siris syndrome

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By exome sequencing, we found *de novo SMARCB1* mutations in two of five individuals with typical Coffin-Siris syndrome (CSS), a rare autosomal dominant anomaly syndrome. As *SMARCB1* encodes a subunit of the SWItch/Sucrose NonFermenting (SWI/SNF) complex, we screened 15 other genes encoding subunits of this complex in 23 individuals with CSS. Twenty affected individuals (87%) each had a germline mutation in one of six SWI/SNF subunit genes, including *SMARCB1, SMARCA4, SMARCA2, SMARCE1, ARID1A* and *ARID1B*.

Chromatin remodeling factors regulate the gene accessibility and expression by dynamic alteration of chromatin structure. SWI/SNF complexes have important roles in lineage specification, maintenance of stem cell pluripotency and tumorigenesis[1–5]. These complexes are composed of evolutionarily conserved core subunits and variant subunits. Brahma-associated factor (BAF) and Polybromo BAF (PBAF) complexes constitute two major subclasses[1–5]. It has been suggested that the BAF complex is similar to the yeast SWI/SNF complex and that the PBAF complex is more like the chromatin remodelling complex (RSC) in yeast, which is required for cell cycle progression through mitosis[6]. However, several subunits that are common to both BAF and PBAF complexes are predicted to be related to the regulation of lineage- and tissue-specific gene expression[2].

Coffin-Siris syndrome (MIM 135900) is a rare congenital anomaly syndrome characterized by growth deficiency, intellectual disability, microcephaly, coarse facial features and hypoplastic nail of the fifth finger and/or toe (**Fig. 1** and **Supplementary Table 1**)[7]. The majority of affected individuals represent sporadic cases, which is compatible with an autosomal dominant inheritance mechanism. The genetic cause for this syndrome has not been elucidated.

To identify the genetic basis of CSS, we performed whole-exome sequencing of five typical affected individuals (**Supplementary Methods**). Taking into account our model that assumes that an abnormality in a causal gene would be shared in two or more subjects, 51 variants were identified as candidates (**Supplementary Table 2**). All the variants were also examined by Sanger sequencing of PCR products amplified using genomic DNA from the five affected individuals and their parents. Nine variants were found to be false positives, 40 were inherited from either the father or mother, and 2 *de novo* heterozygous mutations of *SMARCB1* were found in 2 affected individuals (c.1130G>A (p.Arg377His) and c.1091_1093del AGA (p.Lys364del)) (**Table 1, Supplementary Fig. 1** and **Supplementary Methods**). Two *de novo* coding-sequence mutations occurring within a specific gene is an extremely unlikely event[8], supporting the idea that *SMARCB1* is a causative gene in CSS. Next, we screened *SMARCB1* in 23 individuals with CSS by high-resolution melting analysis[9] and identified the mutation encoding the p.Lys364del alteration in two additional individuals, including one of Arab descent (subject 22) (**Table 1** and **Supplementary Fig. 1**). As the mutation detection rate was relatively low (4 of 23, only 17.4%), we screened 15 additional genes encoding other SWI/SNF subunits (**Supplementary Table 3**). Unexpectedly, four other subunits, *SMARCA4* (also known as *BRG1*), *SMARCE1, ARID1A* and *ARID1B* were also found to be mutated (**Table 1** and **Supplementary Figs. 2–5**). In subject 10, a, c.2144C>T mutation in *ARID1B* (encoding p.Pro715Leu) was found in addition to the c.5632delG mutation in *ARID1B*. RT-PCR products that were amplified from total RNA from this subject's lymphoblastoid cells were cloned into the pCR4-TOPO vector. The two mutations were present on different alleles, according to sequencing of clones containing each allele (data not shown). As the c.5632delG mutation is

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Figure 1 Photographs of individuals with Coffin-Siris syndrome. The faces (left) and hypoplastic-to-absent nail of the fifth finger or toe (right) of affected individuals are shown with the color-coded names of the corresponding mutated genes. The green arrow indicates the absence of the distal phalanx in the fifth toe. No obvious hypoplastic nails were observed in subjects 12 or 19. Consent for all the photographs was obtained from the families of the affected individuals.

in mice[10]. However, in humans, abnormalities in both *SMARCA4* and *SMARCA2* are found in CSS, indicating that the in-frame partial deletion of the gene encoding BRM in subject 19 has a specific mutational effect different from that of simple inactivation in mice. These data support the idea that abnormalities in the BRG1-BAF and BRM-BAF complexes can cause the abnormal neurological development in CSS.

All the mutated genes found in CSS, except for *SMARCE1*, have been reported to be associated with tumorigenesis[1,2]. Among the 23 subjects with CSS, only subject 3 with an *ARID1A* mutation presented with hepatoblastoma. To our knowledge, haploinsufficiency and/or homozygous inactivation of *ARID1A* have been found in several types of cancer but not in hepatoblastoma. Malignancies were not detected in any of the other subjects with CSS examined here. It remains to be seen whether malignancies are robustly associated with CSS.

Given the fact that all the mutations in *ARID1A* and *ARID1B* in CSS were predicted to cause protein truncation, we proposed that haploinsufficiency of these two genes must be able to cause CSS. cDNA analysis of lymphoblastoid cell lines from subjects 1, 6 and 23 indicated that the mutated transcripts were subject to nonsense-mediated mRNA decay (**Supplementary Fig. 8**). In subject 10, the *ARID1B* mutation associated with the creation of a premature stop codon in the last exon did not result in nonsense-mediated mRNA decay as expected (**Supplementary Fig. 8**).

In regard to the other mutated genes, germline heterozygous truncation mutations in *SMARCB1* and *SMARCA4* have been reported

very likely to be deleterious (as it results in a truncated protein), the c.2144C>T mutation is likely to be a rare polymorphism. Of note, subject 12, who presented an atypical facial appearance and indistinct hypoplastic nails, had two interstitial deletions at 6q25.3–q27 involving *ARID1B*, as detected by a SNP array (**Supplementary Fig. 6** and **Supplementary Methods**). Furthermore, subject 14 was found to have an interstitial deletion of *SMARCA2* by a SNP array (**Supplementary Fig. 7** and **Supplementary Methods**). No other copy-number changes involving genes encoding SWI/SNF complex components were found in subjects 2, 14 or 18 by array analysis. The overall mutation detection rate was 87%. In total, 20 of the 23 subjects had a mutation affecting one of the six SWI/SNF subunits.

Mutations in CSS were identified in the BAF-specific subunits *ARID1A* and *ARID1B* but not in PBAF-specific subunits (*BRD7, ARID2* and *PBRM1*) (**Supplementary Table 3**). In addition, mutations were identified in *SMARCA4* (*BRG1*) as well as in *SMARCA2* (*BRM*) (**Supplementary Table 3**). The BRG1 and BRM proteins are mutually exclusive catalytic ATP subunits in mammalian SWI/SNF complexes. Of note, the majority of heterozygous *Smarca4*-null mice survive with susceptibility to neoplasia, with a minority dying after birth because of exencephaly, whereas homozygous *Smarca2*-null mice are viable and fertile[4]. In *Smarca2*-null mice, Brg1 is upregulated, suggesting that Brg1 can functionally replace Brm

Table 1 Mutations in individuals with Coffin-Siris syndrome

Subject ID	Gene	Mutation	Alteration	Type	Control allele frequency[a]
4	SMARCB1	c.1091_1093del AGA	p.Lys364del	De novo	0/502
11	SMARCB1	c.1130G>A	p.Arg377His	De novo	0/500
21	SMARCB1	c.1091_1093del AGA	p.Lys364del	NC	0/502
22	SMARCB1	c.1091_1093del AGA	p.Lys364del	NC	0/502
9	SMARCA4	c.1636_1638del AAG	p.Lys546del	De novo	0/350
7	SMARCA4	c.2576C>T	p.Thr859Met	De novo	0/368
5	SMARCA4	c.2653C>T	p.Arg885Cys	De novo	0/368
16	SMARCA4	c.2761C>T	p.Leu921Phe	De novo	0/368
25	SMARCA4	c.3032T>C	p.Met1011Thr	NC	0/372
17	SMARCA4	c.3469C>G	p.Arg1157Gly	De novo	0/368
19	SMARCA2	Partial deletion		De novo	–
24	SMARCE1	c.218A>G	p.Tyr73Cys	De novo	0/368
3	ARID1A	c.31_56del	p.Ser11Alafs*91	NC	0/330
6	ARID1A	c.2758C>T	p.Gln920*	NC	0/376
8	ARID1A	c.4003C>T	p.Arg1335*	De novo	–
1	ARID1B	c.1678_1688del	p.Ile560Glyfs*89	De novo	–
15	ARID1B	c.1903C>T	p.Gln635*	De novo	–
23	ARID1B	c.3304C>T	p.Arg1102*	De novo	–
10	ARID1B	c.2144C>T	p.Pro715Leu	NC	0/368
10	ARID1B	c.5632del G	p.Asp1878Metfs*96	NC	0/374
12	ARID1B	Microdeletion		NC	–

NC, not confirmed because parental samples were unavailable.
[a]The numbers indicate the observed allele frequency (alleles harboring the change/total tested alleles) in Japanese controls. None of the mutations was found in dbSNP132, the 1000 Genomes database or the National Heart, Lung, and Blood Institute (NHLBI) GO exomesequencing project database. –, not tested.

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BRIEF COMMUNICATIONS

in individuals with rhabdoid tumor predisposition syndromes 1 (RTPS1; MIM 609322) and 2 (RTPS2; MIM 613325)[11,12], and various types of *SMARCB1* mutations (missense, in-frame deletion, nonsense and splice site) have been found in the germline of individuals with familial and sporadic schwannomatosis (MIM 162091)[13,14]. Furthermore, mice with heterozygous knockout of *Smarca4* or *Smarcb1* were prone to tumor development[2]. All the mutations in *SMARCA4* and *SMARCB1* in individuals with CSS were non-truncating (either missense or in-frame deletions), implying that they exert gain-of-function or dominant-negative effects (excluding haploinsufficiency as a cause). It is noteworthy that comparable germline mutations in *SMARCB1* have such different phenotypic consequences in their association with the phenotypes of CSS and schwannomatosis. The *SMARCB1* mutations in CSS and those in schwannomatosis are indeed different according to the Human Gene Mutation Database. With regard to the *SMARCA2* interstitial deletion in CSS, the change maintained the coding sequence reading frame but removed exons 20–27 that encode the HELICc domain. RT-PCR analysis confirmed the deletion of exons 20–27 at the cDNA level (**Supplementary Fig. 7**). These data suggest the importance of the HELICc domain in the SMARCA2 protein.

The various types of mutations in the genes encoding different SWI/SNF components resulted in similar CSS phenotypes. This suggests that the SWI/SNF complexes coordinately regulate chromatin structure and gene expression. This is the first report, to our knowledge, of germline mutations in SWI/SNF complex genes associated with a multiple congenital anomaly syndrome, highlighting new biological aspects of SWI/SNF complexes in humans. Similarly, genes encoding SNF2-related proteins, which are implicated as chromatin remodeling factors outside of SWI/SNF complexes, are mutated in different syndromes, including in α-thalassaemia/mental retardation syndrome X-linked (ATRX; *ATRX* mutations) and in coloboma, heart defect, atresia choanae, retarded growth and development, genital abnormality and ear abnormality (CHARGE) syndrome (*CHD7* haploinsufficiency)[3]. We expect that more mutations affecting chromatin remodeling factors will be found in different human diseases.

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Homozygous c.14576G>A variant of *RNF213* predicts early-onset and severe form of moyamoya disease

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ABSTRACT

Objective: *RNF213* was recently reported as a susceptibility gene for moyamoya disease (MMD). Our aim was to clarify the correlation between the *RNF213* genotype and MMD phenotype.

Methods: The entire coding region of the *RNF213* gene was sequenced in 204 patients with MMD, and corresponding variants were checked in 62 pairs of parents, 13 mothers and 4 fathers of the patients, and 283 normal controls. Clinical information was collected. Genotype-phenotype correlations were statistically analyzed.

Results: The c.14576G>A variant was identified in 95.1% of patients with familial MMD, 79.2% of patients with sporadic MMD, and 1.8% of controls, thus confirming its association with MMD, with an odds ratio of 259 and $p < 0.001$ for either heterozygotes or homozygotes. Homozygous c.14576G>A was observed in 15 patients but not in the controls and unaffected parents. The incidence rate for homozygotes was calculated to be >78%. Homozygotes had a significantly earlier age at onset compared with heterozygotes or wild types (median age at onset 3, 7, and 8 years, respectively). Of homozygotes, 60% were diagnosed with MMD before age 4, and all had infarctions as the first symptom. Infarctions at initial presentation and involvement of posterior cerebral arteries, both known as poor prognostic factors for MMD, were of significantly higher frequency in homozygotes than in heterozygotes and wild types. Variants other than c.14576G>A were not associated with clinical phenotypes.

Conclusions: The homozygous c.14576G>A variant in *RNF213* could be a good DNA biomarker for predicting the severe type of MMD, for which early medical/surgical intervention is recommended, and may provide a better monitoring and prevention strategy. *Neurology®* 2012;78:803-810

GLOSSARY

CI = confidence interval; **HRM** = high-resolution melting; **MMD** = moyamoya disease; **OR** = odds ratio; **PCA** = posterior cerebral artery.

Moyamoya disease (MMD) is a cerebrovascular disease, which is now a relatively common cause of pediatric strokes.[1,2] Annual incidence is estimated to be 0.35–0.54 per 100,000 person-years in Japan[3,4] and about one tenth of that in Europe.[5,6] MMD can lead to devastating neurologic deficits and intellectual impairments if left untreated.

Although MMD is a progressive disease, its natural history varies from slow progression to rapid neurologic decline.[7] Preoperative infarctions, early age at onset, intellectual impairment, seizure, and progressive posterior cerebral artery (PCA) stenosis are known prognostic factors.[8-11] Surgical revascularization can improve the cerebrovascular hemodynamics and prevent subsequent attacks in the ischemic type of MMD.[8] Thus, early diagnosis and surgical intervention are very important.

Genetic factors underlying MMD are of clinical relevance. Epidemiologic studies have shown that about 15% of patients had a family history.[12] Anticipation of the disease is

Table 1	Sample demographics of patients with moyamoya disease and controls[a]		
Clinical features	No. of patients (%)		No. of patients without data
MMD	204		
Gender (M/F)	68 (33.5)/135 (66.5)		1
Distribution of age at onset	0–58 y		7
Frequency of childhood onset (<15 y)	143 (72.6)		
Frequency of childhood onset (<4 y)	36 (18.3)		
Clinical manifestation			11
Infarction	87 (45.1)		
TIA	77 (39.9)		
ICH/IVH	17 (8.8)		
Others	12 (6.2)		
With family history	41 (20.1)		0
With intellectual impairment	33 (17.7)		18
With epilepsy	33 (17.6)		16
PCA involvement			52
Unilateral	31 (20.4)		
Bilateral	43 (28.3)		
Total	74 (48.7)		
Bilateral MMD	148 (96.1)		50
Controls	283		
Gender (M/F)	140 (51.5):132 (48.5)		11

Abbreviations: ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage; MMD = moyamoya disease; PCA = posterior cerebral artery; TIA = transient ischemic attack.
[a] Numbers of patients (%) in each feature are shown, except for the distribution of ages at onset for all patients.

also observed in familial MMD.[13] Recently, the important MMD susceptibility gene, *RNF213*, was identified.[14,15] However, its clinical relevance remains unknown. For this investigation, we conducted a comprehensive genetic study of *RNF213* as well as a clinical phenotype analysis of MMD.

METHODS Study subjects. Blood samples from 204 Japanese patients with MMD were obtained consecutively between January 2008 and February 2011. There were no sample overlaps between ours and those in the previous studies.[14,15] MMD was diagnosed as either definite (bilateral) or probable (unilateral) according to published guidelines.[16] Six patients with probable MMD were female adults and 5 of them had sporadic MMD. The medical charts were completed by the clinicians who were blinded to the genotype of the patients. Sample demographics are shown in table 1. We also obtained either blood or saliva samples from 62 pairs of

parents, as well as 4 fathers and 13 mothers whose partners were unavailable. As many as 94 to 283 samples from healthy Japanese individuals were tested as normal controls for each sequence variant found.

Standard protocol approvals, registrations, and patient consents. Experimental protocols were approved by the Committee for Ethical Issue at Yokohama City University School of Medicine. Written informed consents were obtained from all the patients or their parents.

Mutation screening. Genomic DNA was obtained from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan) or from saliva using Oragene DNA (DNA Genotek, Kanata, Canada). DNA was amplified using GenomiPhi version 2 (GE Healthcare, Buckinghamshire, UK). Mutation analysis of exons and exon-intron borders covering the coding region of *RNF213* (GenBank accession number, NM_020914.4), except for exon 61, was performed in all MMD patient samples by high-resolution melting (HRM) analysis on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Primer sequences, PCR conditions, and HRM settings are available on request. HRM analysis with and without the spike-in method was performed for the detection of homozygous mutations.[17] If samples showed any aberrant melting curve patterns, direct sequencing was performed using an ABI Genetic Analyzer 3100 or 3500xL (Applied Biosystems, Foster City, CA) and analyzed with sequence analysis software version 5.1.1 (Applied Biosystems) and Sequencher 4.10 build 5828 (GeneCodes Corporation, Ann Arbor, MI). For exon 61, which bears the c.14576G>A variant, direct sequencing was performed for all patients with MMD and their parental samples. Additional screenings by HRM analysis were performed for the confirmed mutations in up to 283 normal control Japanese individuals. All variants were confirmed with PCR direct sequencing using either genomic DNA or another DNA, amplified with GenomiPhi separately. No discrepancy was seen in the data between the 2 different conditions of DNA.

Statistical analysis. Patients without information for each clinical feature (listed below) were excluded from the analyses (table 1 and tables e-2 and e-3 on the *Neurology*® Web site at www.neurology.org). All statistical analyses were performed using SPSS Statistics 19 (IBM, New York, NY) software. χ^2 tests were applied to compare each categorical phenotype variable between different genotypes: clinical symptom at onset, with/without family history, intellectual impairment, epilepsy, and the unilateral/bilateral distribution of vasculopathy. Nonnormally distributed continuous variables, such as age at onset and the number of steno-occlusive PCA arteries were compared using the Mann-Whitney U test and Kruskal-Wallis test between different genotypes. $p < 0.05$ was considered statistically significant. A Kaplan-Meier curve was used to assess the cumulative incidence with the log-rank test. The Cox regression model was used to test which variables were associated with age at onset. The exact 95% confidence interval (CI) of the incidence rate of MMD was calculated according to the binomial distribution. The comparisons of clinical features between parent-offspring pairs or sibling pairs were performed using the Wilcoxon signed-rank test and McNemar test.

RESULTS Identification of *RNF213* variants. c.14576G>A was identified in 39 of 41 patients with familial MMD (95.1%), in 129 of 163 patients with nonfamilial MMD (79.2%), and in 5 of

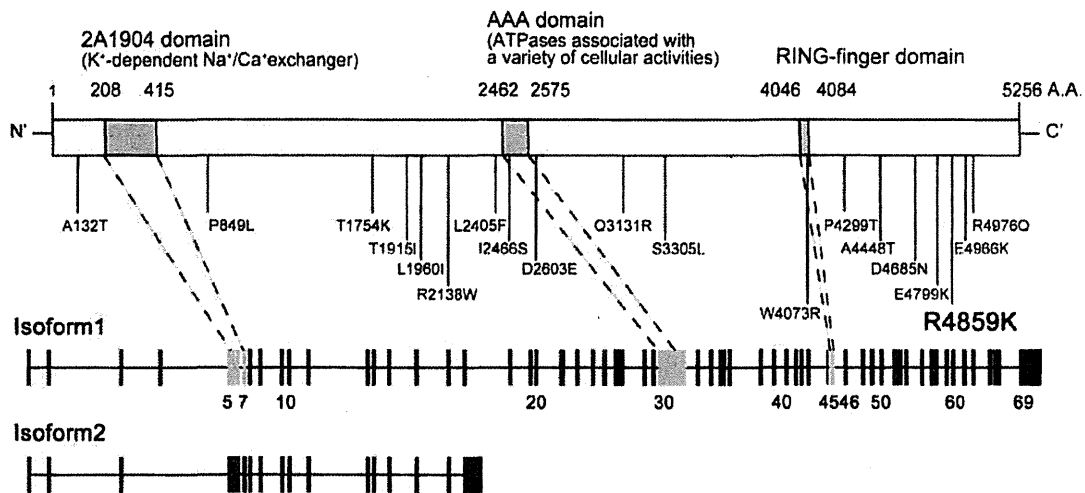| Table 2	Distribution of the c.14576G>A variant among patients with MMD, parents of the patients, and normal control Japanese individuals[a]				
		c.14576G>A genotype			
	Total	Wild-type: G/G (%)	Heterozygous: G/A (%)	Homozygous: A/A (%)	GA + A/A (%)
Patients with MMD	204	36 (17.6)	153 (75.0)	15 (7.4)	168 (82.4)
Sporadic	163	34 (20.8)	117 (71.8)	12 (7.4)	129 (79.2)
With no other variant	137	20 (14.6)	105 (76.6)	12 (8.8)	117 (85.4)
With one other variant	25	13 (52)	12 (48)	0	12 (48)
With one other homozygous variants	1	1 (100)	0	0	0
Familial	41	2 (4.9)	36 (87.8)	3 (7.3)	39 (95.1)
With no other variant	36	0	33 (91.7)	3 (8.3)	36 (100)
With one other variant	3	0	3 (100)	0	3 (100)
With 2 other compound heterozygous variants	2	2 (100)	0	0	0
Parents of patients with MMD	141	77 (54.6)	63 (44.7)	1 (0.7)	64 (45.4)
Affected	9	0	8 (88.9)	1 (11.1)	9 (100)
Unaffected	132	77 (58.3)	55 (41.7)	0	55 (41.7)
Normal controls	283	278 (98.2)	5 (1.8)	0	5 (1.8)
OR (patients with MMD vs normal control)			236	ND	259
95% CI			91-615		100-674
p (Fisher exact test)			<0.001	<0.001	<0.001

Abbreviations: CI = confidence interval; MMD = moyamoya disease; ND = not determined; OR = odds ratio.
[a] Numbers of patients in each category (%) are shown.

283 normal control Japanese individuals (1.8%) (table 2 and table e-1). Sixty-two pairs of parents were also tested for the c.14576G>A genotype, with the conclusion that the c.14576G>A variant allele was inherited from either or both parents in all patients tested. Among 168 patients with the c.14576G>A variant, 15 had a homozygous change, whereas none of the controls and unaffected parents did. We conclude that the heterozygous c.14576G>A variant increases the risk for MMD with an odds ratio (OR) of 236 (95% CI 91–615, $p < 0.001$). Because no homozygous mutation was detected in the control samples or unaffected family members, the OR for the homozygote could not be calculated (∞), suggesting its strong effect. The incidence rate of MMD was calculated to be extremely high with a 95% CI of 0.78–1.00 with the homozygous mutation. Eighteen other genetic variants beside c.14576G>A were also identified in RNF213 (figure 1, table e-1). Sixteen of them were novel, which had not been reported in the previous studies.[14,15] Two of the variants were also found in the previous study[14]; however, they were thought to be common single nucleotide polymorphisms because they were also found in the normal controls without the significant difference of frequency. Other genetic variants showed a relatively small OR without any significance (table e-1). Thirty-one patients had these individual variants (table 2). Fifteen of them also had the heterozygous

c.14576G>A, and 4 of 5 patients whose parents' samples were available had these 2 variants existing as compound heterozygotes (for example, one variant from the father and the other from the mother). In the other 16 patients having no c.14576G>A, 1 had a homozygous c.13342G>A variant, and 2 had 2 variants: c.13342G>A and c.14053G>A as a compound heterozygote. Of the novel 16 variants, 11 of them were not found in 188 normal control Japanese individuals and were all private mutations (only once in one family).

Correlation between the c.14576G>A genotype and clinical phenotype. We compared the clinical features of patients with MMD according to the c.14576G>A genotype, the wild type (genotype GG, as group GG), the heterozygote (genotype GA, as group GA), or the mutant homozygote (genotype AA, as group AA). Age at onset was lower in AA than in GA or GG ($p = 0.002$ or $p = 0.007$) (figure 2A and table e-2). Median age at onset was 3 years in AA, 7 years in GA, and 8 years in GG. Among those with childhood onset (age at onset <15 years), in whom the effect of secondary vascular changes in later life could be ignored and therefore a pure genetic effect could be expected, the association between earlier onset age and the homozygous c.14576G>A genotype was clearly replicated (table e-2). Although the clinical manifestation is different

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A schematic presentation of the RNF213 protein with 3 conserved domains, the genetic variants we have identified, and the genomic structures of 2 *RNF213* isoforms (shown from top to bottom). All missense changes, including R4859K (c.14576G>A as larger characters) are indicated. A.A. = amino acids; AAA = ATPases associated with a variety of cellular activities; RING = really interesting new gene. (Based on National Center for Biotechnology Information Reference sequence, NP_065965.4.)

between the childhood-onset group and the adult-onset group, the rates of the patients with this variant, 83.2% (119 of 143 patients) and 79.6% (43 of 54 patients), respectively, were not significantly different. Among adult patients, there was no significant difference in the rate of having this variant between those with familial history (84.6%, 11 of 13 patients) and those without (78.0%, 32 of 41 patients). The univariate Cox regression analysis showe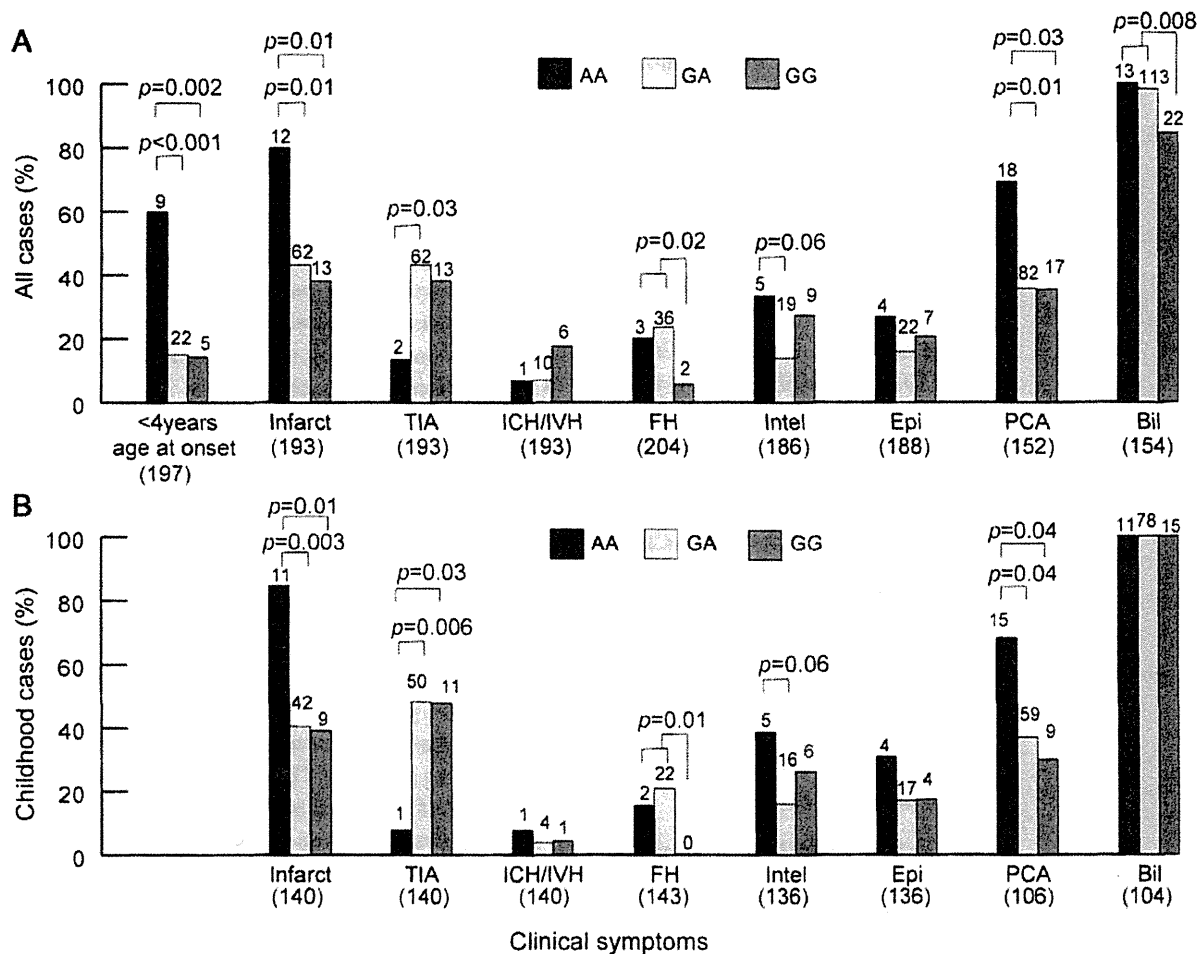d that only the c.14576G>A genotype was the significant predictive variable for age at onset (table e-4). The cumulative incidence of MMD was higher in AA than GA or GG at almost all age distributions (figure 2B), but this tendency was more apparent in the childhood-onset group. Further investigation with more AA and GG patients is necessary for better statistical accuracy. More AA patients were affected before age 4, compared with GA and GG patients ($p < 0.001$) (figure 3A). All AA patients had infarctions at initial presentation.

Figure 2   Correlation between the c.14576G>A variant and age at onset
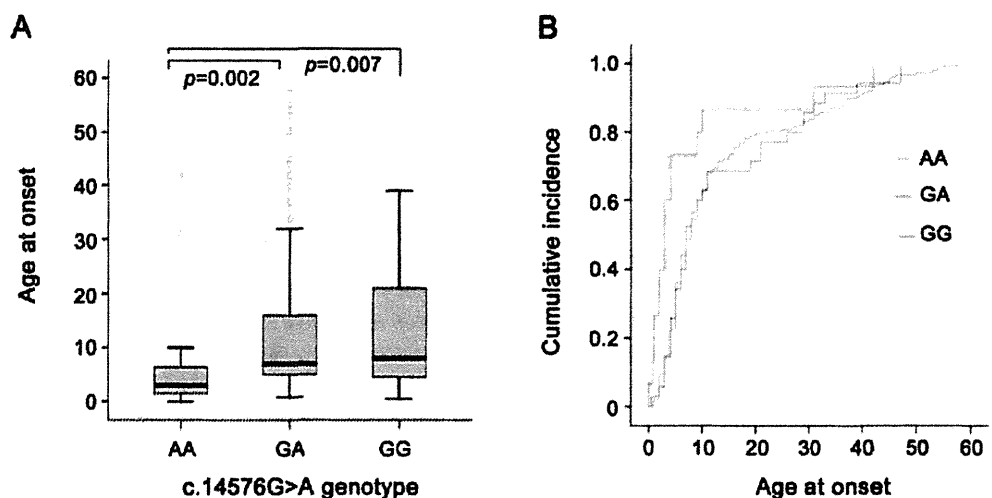
(A) A box plot of age at onset between 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant. O indicates mild outliers; Δ indicates extreme outliners. (B) Cumulative incidence curve of the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant.

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Figure 3　Correlation between c.14576G>A variant and clinical features

A

(y-axis: All cases (%), 0–100)

Bars grouped: AA, GA, GG

- <4years age at onset (197): p=0.002, p<0.001; values 9, 22, 5
- Infarct (193): p=0.01, p=0.01; values 12, 62, 13
- TIA (193): p=0.03; values 2, 62, 13
- ICH/IVH (193): values 1, 1(?), 6
- FH (204): p=0.02; values 3, 36, 2
- Intel (186): p=0.06; values 5, 19, 9
- Epi (188): values 4, 22, 7
- PCA (152): p=0.03, p=0.01; values 18, 82, 17
- Bil (154): p=0.008; values 13, 113, 22

B

(y-axis: Childhood cases (%), 0–100)

Bars grouped: AA, GA, GG

- Infarct (140): p=0.01, p=0.003; values 11, 42, 9
- TIA (140): p=0.03, p=0.006; values 1, 50, 11
- ICH/IVH (140): values 1, 4, 1
- FH (143): p=0.01; values 2, 22, 0
- Intel (136): p=0.06; values 5, 16, 6
- Epi (136): values 4, 17, 4
- PCA (106): p=0.04, p=0.04; values 15, 59, 9
- Bil (104): values 11, 78, 15

Clinical symptoms

(A) The clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant (204 patients). The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. (B) Clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant among those with age at onset younger than 15 years. The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. Bil = bilateral vasculopathy; Epi = epilepsy; FH = with family history; ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage, Infarct = infarction; Intel = intellectual impairment; PCA = posterior cerebral artery involvement.

The frequencies of other clinical features of MMD in AA, GA, and GG were also compared (figure 3A and table e-2). As the clinical manifestation at diagnosis, infarction was more common in AA than in GA or GG ($p = 0.01$, OR 5.3, 95% CI 1.43–19.56 or $p = 0.01$, OR 6.5, 95%CI 1.53–27.32); TIA was less common in AA than in GA ($p = 0.03$; OR 0.20; 95% CI 0.04–0.94). Bilateral MMD and family history of the disease were more frequent in AA and GA than in GG ($p = 0.008$, OR 11, 95% CI 1.98–66.36 and $p = 0.02$, OR 5.1, 95% CI 1.18–22.36). The number of steno-occlusive PCAs was larger in AA than in GA ($p = 0.01$) (counted as 2 arteries per person). Seventy-four of the 152 patients (48.6%) had PCA lesions, and infarctions and intel-lectual impairment were more frequent in those with PCA involvement than those without (infarctions 68.9% vs 30.8%, $p < 0.001$; intellectual impairment 26.8% vs 5.3%, $p < 0.001$). Intellectual impairment and epilepsy tended to be a more common complication in AA than in GA, with and without marginal significance. We also compared these clinical features in AA, GA, and GG, excluding 6 patients with uni-lateral MMD, but the results were not changed (data not shown). Among childhood-onset cases (age at onset <15 years), the associations between the c.14576G>A genotype and these clinical features were generally similar, except for bilateral vasculopathy (all genotypes in childhood-onset cases showed bilateral involvement) (figure 3B).

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Correlation between variants other than c.14576G>A and clinical phenotype. We also compared the clinical features of patients with MMD with the other variants, except the c.14576G>A variant, with those without (table e-3). Interestingly, none of the c.14576G>A homozygotes had any other variants. The other patients were categorized into 4 groups, who showed at least one of any individual variants without c.14576G>A (as group GG1), no other variant without c.14576G>A (as group GG0), at least one of any other variants with heterozygous c.14576G>A (as group GA1), and no other variant with heterozygous c.14576G>A (as group GA0). Although there were no differences in age at onset between GG1 and GG0 patients, it was lower in GA0 patients than GA1 patients ($p = 0.03$). Median age at onset was 7 years for GA0 and 12 years for GA1. The frequency of infarctions was lower and that of intracerebral hemorrhage was higher in GA1 than in GA0 ($p = 0.02$, OR 0.19, 95% CI 0.04–0.90 and $p = 0.009$, OR 8.3, 95% CI 2.00–34.19). However, when patients with MMD with another variant, which was predicted to be pathogenic by PolyPhen-2[18] or SIFT[19] algorithms, were compared with those without, consistently no differences in any of these clinical features were observed (data not shown). Further analyses with larger numbers of patients are needed to validate this effect.

Anticipation of MMD. In addition, statistical comparisons of clinical features between 5 parent-offspring pairs having the same RNF213 genotype (heterozygous c.14576G>A) were performed (table e-5). Age at onset was lower in the second generation than in the first generation ($p = 0.04$). Median age at onset was 5 and 37 years, respectively. This result may support the anticipation of MMD as reported previously.[13] Conversely, age at onset was not different between 6 sibling pairs having the same RNF213 genotype ($p = 0.67$). Median age at onset was 8 years for the older siblings and 12.5 years for the younger ones. There were no differences in other clinical symptoms among patients from the same pedigree.

DISCUSSION We confirmed a strong association between c.14576G>A in RNF213 and MMD with the larger number of Japanese patients different from those of the previous studies.[14,15] More importantly, this is the first report showing the significant phenotype-genotype correlation. The OR for the heterozygous c.14576G>A was 236 ($p < 0.001$) and could not be exactly calculated for the homozygote (∞). With the assumption that the effects of both heterozygous and homozygous changes on MMD onset were similar, the homozygous

c.14576G>A variant would increase the risk with an OR of 259 (95% CI 100–674, $p < 0.001$). However, the effect of the homozygous variant on MMD onset was expected to be much larger than that of the heterozygote because no homozygote was found in a total of 283 normal controls and 132 unaffected family members in this study and 429 normal controls and 28 unaffected family members in the previous study.[14] We also showed that the risk of being diagnosed with MMD with the homozygous variant was more than 78%. Although this variant does not exactly fit the pure Mendelian inheritance pattern because it is observed to some extent in the normal population, this variant might have a much larger effect on the pathogenesis of MMD than the common variants of complex diseases, considering its extremely high OR. This rare variant could be an example of missing heritability, that is, the majority of heritability of complex traits that are unexplained by common variants with a small effect size.[20,21] Thus, this variant should not be considered as one of common variants contributing to common diseases.

The c.14576G>A variant has not been found among the total number of 55 Caucasian patients in the previous studies on RNF213.[14,15] However, 4 other rare variants were identified in 4 of 50 Caucasian patients.[15] The overall variant detection rate for RNF213 was as high as 90.2% for our Japanese patients, in contrast to 8% for the Caucasian patients in the previous study.[15] Importantly, 82.4% of our patients were accounted for by the c.14576G>A variant. It was reported that c.14576G>A variant was identified in 90% of Japanese patients, 79% of Korean patients, and 23% of Chinese patients.[15] The founder effect widely distributed in some areas of east Asia was likely to be expected, and this variant could explain the difference of prevalence of MMD between Asian and non-Asian populations.

RNF213 is a RING (really interesting new gene) finger protein containing an AAA (ATPases associated with variety of a cellular activities) domain, indicating that it has E3 ubiquitin ligase activity and energy-dependent unfoldase activity.[14,22] Knockdown of RNF213 in zebrafish leads to the abnormal sprouting and irregular diameter of intracranial vessels, suggesting its possible contribution to vascular formation.[15] More research on its contribution to MMD pathogenesis will be necessary.

Although the number of adult-onset cases was relatively small, the similar rates of the cases with this variant between childhood-onset patients and sporadic adult-onset patients might suggest that the variant apparently contributes to both groups. Either a heterozygous or homozygous c.14576G>A variant increased the risk for adult-onset MMD (OR 217,

95% CI 72–656, $p < 0.001$) compared with that in adult normal controls.

Whether bilateral and unilateral MMD belong to a single entity is a very important question. Of the 6 patients with unilateral MMD, 2 were heterozygotes and the others were wild types, which indicated a lower frequency of heterozygotes than that in the previous study.[14] Because we showed a significant difference in the frequency of bilateral vasculopathy between GG and other genotypes, we speculate that to some extent patients with unilateral MMD share a genetic background, but there could be different genetic backgrounds in these groups. Further investigation is needed to confirm these findings with larger numbers of patients with unilateral MMD.

The recent spread of brain check-up has increased the opportunity to encounter patients with asymptomatic MMD.[23] Whereas our patients in this study all had symptomatic MMD, it is necessary to further examine the RNF213 variant in the asymptomatic group.

The homozygous c.14576G>A variant carriers showed significantly earlier age at onset, more frequent occurrence of infarctions at initial presentation, and PCA involvement. The association of PCA involvement and infarction or intellectual impairment in our data were compatible with the previous report.[11] These features indicate that c.14576G>A homozygotes have more severe and wider vasculopathy in the brain. The other poor prognostic factors, such as intellectual impairment and epilepsy,[8] were probably more frequent in homozygotes but did not reach statistical significance. We speculated that these conditions might be modified or prevented by early diagnosis and by surgical and medical interventions.

Early surgery for young patients with MMD (<3–4 years of age) has been recommended previously,[24] because they often demonstrate a more severe clinical course.[9,10,24] Approximately 80% of these patients had infarction at initial presentation and had subsequent preoperative infarctions more frequently than patients with older age at onset.[24,25] In our study, 77.1% of the patients diagnosed before age 4 had infarctions at diagnosis, whereas 38% of those diagnosed after age 4 had infarctions ($p < 0.001$), results similar to the previous data.

Conversely, it was demonstrated that young age at onset of symptoms did not always herald a poor later outcome. Instead, neurologic deficits due to infarctions at the time of surgery held the most prognostic value.[7,26] It was recently reported that an irreversible infarction was the greatest risk for an unfavorable outcome by multivariate logistic regression analysis.[8] Specific biomarkers, which might be strongly associated with infarction, would be of invaluable clinical importance to provide the appropriate timing for an operation. In our study, 60% of homozygous c.14576G>A individuals were diagnosed with MMD before age 4, and all of them had infarctions at initial presentation. Thus, the homozygous c.14576G>A variant may be a more specific predictor, which would discriminate those with poor prognosis from those with relatively favorable prognosis among patients with young-onset MMD.

We therefore propose that the homozygous c.14576G>A genotype could be an efficient DNA marker predicting the severe type of MMD with a poor prognosis and a strong biomarker for patients requiring early operation. c.14576G>A genotyping could also be useful to predict the actual risk of severe initial infarctions. Careful follow-up of these high-risk homozygotes could make it possible to undertake intervention before the first infarctions and prevent the irreversible neurologic deficits that can occur in these patients. Thus, the homozygous c.14576G>A variants may provide a better monitoring and prevention strategy. Furthermore, this variant could be very useful in genetic counseling.

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Fused teeth, macrodontia and increased caries are characteristic features of neurofibromatosis type 1 patients with *NF1* gene microdeletion

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Abstract. Neurofibromatosis type 1 (NF1) is the most common genetic condition caused by *NF1* gene alteration. A 1.5 Mb submicroscopic deletion encompassing the entire *NF1* gene, is known to be responsible for approximately 5% of NF1 cases. Patients with *NF1* deletion, compared to those with *NF1* mutation tend to exhibit more severe phenotypes. To know the possible differences in oral/dental features between *NF1* deletion and *NF1* mutation patients, we examined four patients with *NF1* deletion and three with *NF1* mutation to compare their oral manifestations. Fused teeth in the mandibular anterior region were found only in the patients with deletion (2/4). Macrodontia was noted in all four patients with an *NF1* deletion. Although macrodontia was also found in one patient with a mutation, it was relatively mild compared to the deletion patients. Dental caries were observed in both *NF1* deletion (4/4) and mutation (2/3) patients. However, patients with *NF1* deletions showed more apparently severe caries (average number of dental caries 12.8) than those with *NF1* mutation (average number 5.5). Other features also noted in patients with both deletions and mutations were high-arched palate, hypodontia and malocclusion. Our study might suggest that fused teeth, macrodontia and increased dental caries are distinctive manifestations of *NF1* deletion. Providing comprehensive dental care from early infancy would be very important to prevent dental caries especially in patients with *NF1* deletion.

Keywords: Neurofibromatosis type 1, *NF1* gene deletion, fused teeth, macrodontia, caries

1. Introduction

Neurofibromatosis type 1 (NF1) is the most common autosomal dominant genetic condition caused by *NF1* gene alteration. It affects 1 in 3,000 individuals and is characterized by multiple café au lait spots, neurofibromas, and axillary/inguinal freckling [1,2]. Other features associated with the disease include iris Lisch nodules, optic glioma, skeletal dysplasia, plexiform neurofibromas, and mental retardation/learning disability. Although tumors in the oral and facial areas have been frequently reported in association with NF1, the dental manifestations have not been fully characterized so far. To our knowledge, impacted teeth, displaced teeth, missing teeth, supernumerary teeth, increased dental caries, early primary tooth eruption, malocclusion, and periapical cemental dysplasia (in adult female patients) have been reported in patients with NF1 [3–7].

A submicroscopic deletion which is usually 1.5 Mb in size and involves the entire *NF1* gene and more than 20 genes adjacent to *NF1* gene is known to be responsible for approximately 5% of NF1 cases [8,9]. Patients

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with *NF1* deletions, compared to those with *NF1* mutations, tend to exhibit characteristic phenotypes such as facial dysmorphism, mental retardation and learning disability, plexiform neurofibromas, skeletal anomalies and cardiovascular defects, likely due to the involvement of contiguous genes around *NF1* [8,10–14]. However, the possible differences in oral/dental features between *NF1* deletion and *NF1* mutation patients have not been investigated in detail. We examined four patients with *NF1* deletion and three with *NF1* mutation to compare their oral manifestations.

2. Materials and methods

2.1. Patients

A total of seven patients were examined at Saitama Children's Medical Center; four (one male, three females; aged 5–12 yr) were identified as having a microdeletion including the *NF1* gene, and three (two males, one female; aged 5-12 yr) were identified as having a mutation of the *NF1* gene. Microdeletions were analyzed by fluorescence in situ hybridization analysis of metaphase chromosomes from peripheral blood, using a total of seven bacterial artificial chromosome clones comprising the bacterial artificial chromosome clone including *NF1* (RP11-876L22) and six neighboring clones (RP11-96L17, RP11-946G8, RP11-525H19, RP11-278E4, RP11-164M10, and RP11-55J8). The results showed that a deletion of approximately 1.5 Mb

was detected in all four patients. Mutation analysis using genomic DNA extracted from peripheral blood was performed by means of polymerase chain reaction and direct sequencing of the coding regions for all exons. The results identified a splice mutation (c.1185+1G<A) in patient 5 and nonsense mutations (c.574C>T and c.3986C>G, respectively) in patients 6 and 7. Clinical manifestations are shown in Table 1. Neurofibromas of the oral and maxillofacial region were not present in any patient. This study protocol was approved by the Ethics Committee of Saitama Children's Medical Center and proper informed consents were obtained from the patients and their legal guardians of the patients.

2.2. Examination of craniofacial and oral condition by dental casts and radiographs

Palate morphology, occlusion, tooth size, and dental arch were evaluated by intraoral examination and dental cast studies. The relationship of the skeletal and dental structures and congenital hypodontia were evaluated on lateral cephalograms and orthopantomographs. The dimensions of the crown and dental arch were measured using a caliper with a resolution accuracy of 0.01 mm. Lateral cephalometric analysis was performed based on the method developed by Iizuka and Ishikawa [15] (Fig. 1). All data in this study (tooth size, dental arch form size, cephalometric findings) were compared with normal values in Japanese individuals.

Table 1
Clinical manifestations of the seven patients with neurofibromatosis type 1

| Patients	Deletion				Mutation		
	1	2	3	4	5	6	7
Gender	F	M	F	F	M	F	M
Age (Years)	12	5	5	6	12	5	6
Height (SD)	−0.68	1.02	−0.41	0.57	−1.02	−1.50	−0.08
Occipito-frontal circumference	−1.27	1.90	0.31	0.50	0.00	0.29	2.80
Mental retardation	−	+	+	−	−	−	−
Facial dysmorphism	+	+	+	+	−	+	−
Café au leit spots	+	+	+	+	+	+	+
Neurofibroma	+	+	−	−	−	+	−
Plexifrom neurofibroma	−	−	−	−	−	+	−
Optic glioma	−	−	−	−	+	−	−
Brain MRI	UBO	UBO	UBO	UBO	UBO astrocytoma	UBO	UBO
Others		Calcifying epithelioma	VUR, urachal cyst	Preauricular tag			Pes planovalgus
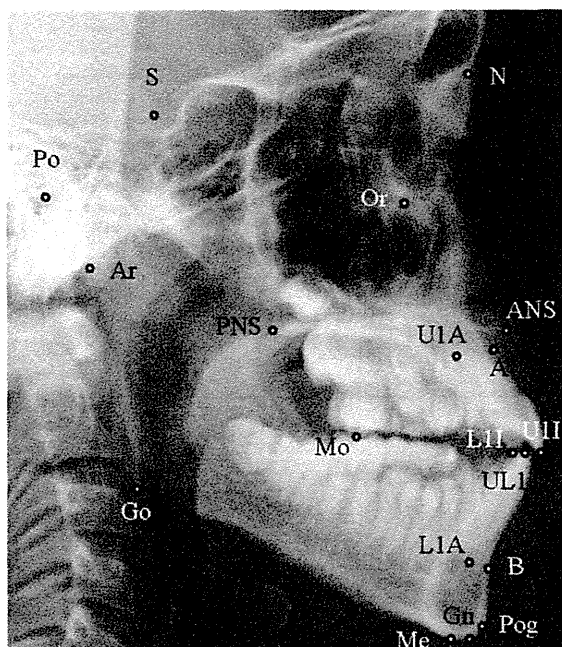
Fig. 1. Landmark points, angles and lines used in cephalometric analysis. Landmarks: N = Nasion; Or = Orbitale; S = Sella turcica; Po = Porion; Ar = Articulare; Go = Gonion; Me = Menton; Gn = Gnathion; Pog = Pogonion; B = B-point; A = A-point; ANS = Anterior nasal spine; Mo = Molar occlusion; U1A = Upper central incisor root apex; U1I = Upper central incisor edge; L1A = Lower central incisor root apex; L1I = Lower central incisor edge; UL1I = Middle of U1I and L1I. Angles: Convexity = N-A line to the A-Pog line; A-B plane = N-Pog line to the A-B line; SNA = S-N line to the N-A line; SNB = S-N line to the N-B line; Facial angle = Po-Or line to the N-Pog line; SNP = S-N line to the N-Pog line; Y axis = Po-Or line to the S-Gn line; SN-S.Gn = S-N line to the S-Gn line; Mandibular plane = Po-Or line to the Me-the lower border of the mandible line; Gonial angle = Ar-the posterior border of the ramus of the mandible line to the Me-the lower border of the mandible line; GZN = S-N line to the Ar-the posterior border of the ramus of the mandible line; FH to SN = Po-Or line to the S-N line; U-1 to FH plane = U1I-U1A line to the Po-Or line; U-1 to SN plane = U1I-U1A line to the S-N line; L-1 to mandibular = L1I-L1A line to the Me-the lower border of the mandible; Interincisal = U1A-U1I line to the L1A-L1I line; Occlusal plane = Po-Or line to the Mo-UL1I line.

3. Results

Oral manifestations noted in seven patients are summarized in Table 2. The prevalence of high-arched palate was high in both the NF1 deletion and mutation patients (deletion 3/4, mutation 2/3). Dental caries occurred more frequently in all four patients with NF1 deletion and two of the three patients with NF1 mutation, but tooth decay was more severe in the patients with deletion compared with those with mutation, with an average of 12.8 affected teeth [10–16] in the former and 5.5 teeth (4

and 7) in the latter type. While mutation type patients showed caries only in the posterior teeth with less dental plaque, deletion type patients had caries both in the anterior and posterior teeth and also had more dental plaque as well (Fig. 2). Fused teeth were present in the mandibular anterior region in two out of four of the patients with NF1 deletion (Patients 2 and 3). Panoramic radiographs showed congenital hypodontia of the permanent teeth in both these patients (Patient 2: bilateral second mandibular premolars and left mandibular first premolar; Patient 3: bilateral maxillary and mandibular second premolars and left mandibular incisor), with hypodontia of the succeeding permanent teeth for the fused teeth in patient 3. In the patients with NF1 mutation, hypodontia (bilateral maxillary second premolars) was present in one out of three patients (Fig. 3). In terms of tooth size, macrodontia was present in all four NF1 deletion patients and in one out of three mutation patients. The number of teeth greater than 2 SD larger than normal averaged 7.8 in patients with NF1 deletion (4–13 teeth). Only three teeth exhibited macrodontia in the single NF1 mutation patient (Table 3). Malocclusion was present in one out of four patients with NF1 deletion (Patient 1: crowding) and one of the patients with NF1 mutation (Patient 6: open bite). The dental crowding in patient 1 (deletion type) was associated with the patient's narrow dental arch and severe macrodontia (Table 4). In patient 6 (mutation type), it was judged to be caused by tongue thrusting. Lateral cephalometric analysis showed a tendency toward a dolichofacial pattern in the patients with NF1 deletion with maxillary protrusion, and a tendency toward labioclination of the maxillary central incisors in those with NF1 mutation (Table 5).

4. Discussion

The intraoral characteristics seen commonly in both deletion and mutation patients studied were high-arched palate, hypodontia, macrodontia, malocclusion and increased dental caries. Fused teeth were found only in the patients with deletion. Fusion of teeth is a relatively rare dental anomaly observed in the general population at a frequency of 4.10% [16]. Therefore, the fact that we observed fused teeth in two of four patients with NF1 deletion suggests that it may be a characteristic feature of NF1 deletion. It is noteworthy that both patients (Patients 2 and 3) who exhibited hypodontia had fused teeth. Macrodontia was noted in all four patients with NF1 deletion. Macrodontia is a rare dental anomaly which may occur in isolation or