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Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

Hitoshi Hiura[1], Hiroaki Okae[1], Naoko Miyauchi[1], Fumi Sato[1],
Akiko Sato[1], Mathew Van De Pette[2], Rosalind M John[2],
Masayo Kagami[3], Kunihiko Nakai[4], Hidenobu Soejima[5],
Tsutomu Ogata[6], and Takahiro Arima[1],*

[1]Department of Informative Genetics, Environment and Genome Research Center, Tohoku University Graduate School of Medicine, 2-1
Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan [2]Cardiff School of Biosciences, Museum Avenue, Cardiff CF10 3US, UK [3]Division of Clinical
Genetics and Molecular Medicine, National Center for Child Health and Development, 2-10-1 Okura, Seatagaya-ku, Tokyo 157-8535, Japan
[4]Department of Development and Environmental Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[5]Division of Molecular Genetics and Epigenetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga
849-8501, Japan [6]Department of Pediatrics, Faculty of Medicine, Hamamatsu University, Hamamatsu 431-3192, Japan

*Correspondence address. Tel: +81-22-717-7844; Fax: +81 22-717-7063; E-mail: tarima@med.tohoku.ac.jp

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

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Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes.

Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

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. S1). 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 *KCNQ1OT1(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

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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Methylation screening of reciprocal genome-wide UPDs identifies novel human-specific imprinted genes[†]

Kazuhiko Nakabayashi[1,‡,¶], Alex Martin Trujillo[3,¶], Chiharu Tayama[1], Cristina Camprubi[3], Wataru Yoshida[1], Pablo Lapunzina[4], Aurora Sanchez[5], Hidenobu Soejima[6], Hiroyuki Aburatani[7], Genta Nagae[7], Tsutomu Ogata[2], Kenichiro Hata[1] and David Monk[3,*,‡]

[1]Department of Maternal-Fetal Biology and [2]Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan, [3]Cancer Epigenetic and Biology Program (PEBC), Institut d'Investigació Biomedica de Bellvitge (IDIBELL), Hospital Duran i Reynals, Barcelona, Spain, [4]Instituto de Genética Médica y Molecular (INGEMM), CIBERER, IDIPAZ-Hospital Universitario La Paz, Universidad Autónoma de Madrid, Madrid, Spain, [5]Servei de Bioquímica i Genètica Molecular, CIBER de Enfermedades Raras, and Institut d'Investigacions Biomèdiques August Pi i Sunyer, Hospital Clínic, Barcelona, Spain, [6]Division of Molecular Genetics and Epigenetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga 849-8501, Japan and [7]Genome Science Division, Research Center for Advanced Science and Technology, the University of Tokyo, Tokyo 153-8904, Japan

Nuclear transfer experiments undertaken in the mid-80's revealed that both maternal and paternal genomes are necessary for normal development. This is due to genomic imprinting, an epigenetic mechanism that results in parent-of-origin monoallelic expression of genes regulated by germline-derived allelic methylation. To date, ~100 imprinted transcripts have been identified in mouse, with approximately two-thirds showing conservation in humans. It is currently unknown how many imprinted genes are present in humans, and to what extent these transcripts exhibit human-specific imprinted expression. This is mainly due to the fact that the majority of screens for imprinted genes have been undertaken in mouse, with subsequent analysis of the human orthologues. Utilizing extremely rare reciprocal genome-wide uniparental disomy samples presenting with Beckwith–Wiedemann and Silver–Russell syndrome-like phenotypes, we analyzed ~0.1% of CpG dinculeotides present in the human genome for imprinted differentially methylated regions (DMRs) using the Illumina Infinium methylation27 BeadChip microarray. This approach identified 15 imprinted DMRs associated with characterized imprinted domains, and confirmed the maternal methylation of the *RB1* DMR. In addition, we discovered two novel DMRs, first, one maternally methylated region overlapping the *FAM50B* promoter CpG island, which results in paternal expression of this retrotransposon. Secondly, we found a paternally methylated, bidirectional repressor located between maternally expressed *ZNF597* and *NAT15* genes. These three genes are biallelically expressed in mice due to lack of differential methylation, suggesting that these genes have become imprinted after the divergence of mouse and humans.

INTRODUCTION

Genomic imprinting is an epigenetic process in which one allele is repressed, resulting in parent-of-origin specific monoallelic expression (1). To date, around 100 imprinted transcripts have been identified in mouse, including protein coding genes, long non-coding RNAs (ncRNA) and microRNAs. Approximately two-thirds show conserved imprinting status between mouse and humans, while some show imprinting restricted to humans (http://igc.otago.ac.nz/home.html).

Genomic imprinting is regulated by epigenetic modifications such as DNA methylation, along with repressive histone modifications that are transmitted through the gametes from the parental germlines (1). Many imprinted regions contain differentially methylated regions (DMRs) that exhibit parent-of-origin-dependent DNA methylation. Of the 21 known DMRs in mouse, a subset have been shown to function as cis-acting imprinting control regions (ICRs) orchestrating the monoallelic expression of genes over more than 100 kbp away (2). The establishment of imprinted methylation in both the maternal and paternal germlines requires the de novo DNA methyltransferae Dnmt3a and its related protein Dnmt3L (3,4). Maintenance of these DMRs is stable throughout somatic development and is regulated by Dnmt1 and Uhrf1 during DNA replication (5,6).

The identification of novel imprinted genes is important as it is becoming increasing evident that alterations in the fine-tuning of imprinted gene expression can influence a number of complex diseases such as obesity, diabetes, neurological diseases and cancer (7–9), in addition to the well-defined imprinting syndromes associated with severe disruption of imprinted domains.

The identification of imprinted genes has traditionally been performed in mouse owing to the ease of embryo and genetic manipulations, and has utilized gynogenetic and androgenetic embryos, or mice harboring regions of uniparental disomy (UPD), where two copies of an entire chromosome or chromosomal region is inherited from only one parent (reviewed in 10). These embryos have then been used in expression screen-based approaches such as subtractive hybridization, differential display or expression array hybridization (11,12). However, these screens are not deemed comprehensive, as imprinted gene expression can be both tissue- and developmental-stage specific. Previously, sophisticated screens have detected allelic differences in DNA methylation at imprinted DMRs present in all somatic tissues, irrespective of temporal and spatial expression. Techniques such as restriction landmark genomic screening, methylation-sensitive representation difference analysis (Me-RDA) and methylated DNA immunoprecipitation (MeDIP) have identified regions of allelic DNA methylation associated with chromosomal regions controlling several imprinted genes in mice (13–15) and humans (16,17).

In order to identify novel imprinted genes in humans, we have performed a quantitative genome-wide methylation screen comparing the methylomes of three-genome-wide paternal UPD (pUPD) samples identified with Beckwith–Wiedemann-like phenotypes and one genome-wide maternal UPD (mUPD) Silver–Russell-like syndrome case (18–21)

with the methylomes of six normal somatic tissues. The genome-wide UPD samples were all mosaic, and we utilized DNA extracted from leukocytes as these presented with lowest level of the biparental cell line. The DNA methylation profiles of these samples only differ at imprinted DMRs, since they are all derived from leukocytes, making them ideal to screen for novel imprinted loci. We utilized the Illumina Infinium Human Methylation27 BeadChip microarray and were able to identify 15 imprinted DMRs associated with known imprinted transcripts, and confirm the allelic methylation within intron 2 of the *RB1* gene (22).

By comparing the methylation profiles of six somatic tissues and the genome-wide UPD cases, we identified a novel paternally methylated DMR which acts as a directional silencer resulting in the maternal expression of *ZNF597* (also known as *FLJ33071*) and *NAT15* on chromosome 16, and a maternally methylated DMRs encompassing the promoter region of the *FAM50B* retrotransposon on chromosome 6, which is paternally expressed in human tissues. Interestingly, the CpG islands of the mouse orthologues of *ZNF597*, *NAT15* and *FAM50B* are all unmethylated, resulting in biallelic expression in mid-gestation embryonic tissues.

RESULTS

Defining a hemimethylated data set

Almost all imprinted domains contain at least one region of allelic DNA methylation which is thought to regulate imprinting *in cis* (1). In order to identify new imprinted genes in humans, we performed a methyation screen of six different normal somatic tissues derived from the three germinal layers (placenta, leukocytes, brain, muscle, fat, buccal cells) and compared the data set with the methylation profiles from reciprocal genome-wide UPD samples. Genomic DNA was modified by sodium bisulfite treatment and hybridized to the Illumina Infinium Human Methylation27 platform. This array covers 27 578 CpG dinucleotides associated with 14 000 human genes. To identify novel imprinted DMRs, we took advantage of the fact that these CpG-rich sequences have a methylation profile of ~50% in all somatic tissues. We identified 78 CpG probes associated with 15 known imprinted DMRs on the array (average methylation 52%, SD 11.7) (Supplementary Material, Fig. S1). To define a range in which novel imprinted DMRs should lie, we used the mean for the known imprinted DMR ± 1.5 SD (range 34.4–69.6). After applying these defined cutoffs, we identified 3212 CpG probes for which the mean methylation value for all normal tissues was within this range. To rule out the possibility that a mean of ~52% was caused by extreme values of hyper- and hypo-methylation as a result of tissue-specific methylation, we only assessed those within 1.8 times SD distance from the methylation average. This step ensures that the ~52% methylation value is representative of all tissues. Using these criteria, we reduced the data set to 1836 CpG probes, which were in addition to 72 probes mapping to known imprinted DMRs.

Determining the allelic methylation using genome-wide UPDs

To identify novel imprinted DMRs within the above hemi-methylated data set outlined earlier, we compared the tissue methylation profiles to those obtained for the samples with genome-wide UPD. Of the 1836 CpG probes, only 14 gave methylation profiles consistent with an imprinted profile (Supplementary Material, Fig. S2). We subsequently mapped the exact location of the candidate CpGs using the genomic sequence of the unconverted DNA probes in the BLAT search tool (UCSC Genome Bioinformatics http://genome. ucsc.edu/). These 14 CpG probes were located close to nine autosomal genes, *RB1* (5), *FAM50B* (2), *ZNF597* (1), *TRPC3* (1), *SYCE1* (2), *TSP50* (1), *SORD* (1) and *ZBTB16* (1). We identified five independent probes located throughout CpG 85 (the CpG island identifier on the UCSC genome browser, build GRCh37/hg19) of the recently identified *RB1* imprinted gene on chromosome 13. These probes were unmethylated with average β-values of 0.21, 0.17 and 0.18 in the three genome-wide pUPD samples but hypermethylated, having an average β-value 0.88, in the genome-wide mUPD sample (a complete unmethylated CpG has a β-value of 0, and a fully methylated dinucleotide being 1). Using bisulphite PCR incorporating the single-nucleotide polymorphism (SNP) rs2804094 and sequencing of individual DNA strands, we were able to confirm that this 1.2 kb CpG island is a maternally methylated DMR in placenta, leukocyte and kidney-derived DNA and unmethylated in sperm (Supplementary Material, Fig. S3).

We identified one probe was located close to CpG 55 of the *TRPC3* gene on human chromosome 4 that was suggestive of a maternally methylated DMR. Subsequent allelic bisulphite PCR encompassing the SNP rs13121031 revealed that this region was subject to SNP-associated methylation and not parent-of-origin methylation (data not shown). The CpG islands within the promoters of *ZBTB16*, *TSP50* and *SORD* each had one probe that was suggestive of imprinted methylation, however allele-specific bisulphite PCR analysis revealed that these regions had a mosaic methylated profile (data not shown).

Two probes mapping to CpG 124 of *SYCE1/SPRNP1* on chromosome 10 also had a methylation profile consistent with an imprinted DMR. However, these probes were unable to discriminate *SYCE1* from *SPRN*, a second region that shared 93% homology. Due to the difficulty in designing bisulphite PCR primers that could specifically target *SYCE1*, we were unable to validate our initial observations.

The *ZNF597/NAT15* CpG island is a paternally methylated DMR

To date, only seven paternally methylated DMRs have been identified, the somatic DMRs at the *NESP, IGF2-P0* and *MEG3/GTL2* promoters, the germline *H19* differentially methylated domain (DMD), *Rasgrf1* DMD, IG-DMR and *ZDBF2* DMR (15,23–26). The *RASGRF1* is not imprinted in humans due to lack of the DNA repeat elements that are involved in establishing germline methylation (27). We identify two CpG probes, one mapping to CpG 41 between the promoters of *ZNF597* and *NAT15*, the other 500 bp away, in

a region flanking CpG 41. Both probes were hypermethylated in the three genome-wide pUPD samples (β-values of 0.83, 0.42, 0.75) and hypomethylated (β-value of 0.08) in the genome-wide mUPD sample. Using bisulphite PCR and subsequent sequencing of heterozygous DNA samples for the SNP rs2270499, we were able to confirm that the methylation was solely on the paternally derived allele in placenta, leukocyte and kidney (Fig. 1). This is consistent with the previous report that *ZNF597* is maternally expressed in human leukocytes (28). Bisulphite PCR and sequencing of sperm DNA revealed that this region lack methylation, indicating that CpG41 is not a germline DMR. Using allele-specific RT–PCR that incorporated coding SNPs within exon 3, we observed maternal expression in brain ($n = 1$) and placenta ($n = 3$), and confirmed imprinting in leukocytes ($n = 2$).

The gene encoding *N*-acetyltransferase 15, *NAT15*, is encoded by two different transcripts (Fig. 1A). To determine whether *NAT15* is also subject to genomic imprinting, we performed allelic RT–PCR using PCR primers that could discriminate each isoform. We find that *NAT15* isoform 1 is maternally expressed in both placenta ($n = 5$) and leukocytes ($n = 1$), whereas isoform 2 is biallelically expressed ($n = 4$) which is consistent with CpG 101 being unmethylated (Fig. 1, data not shown).

FAM50B DMR shows graduated methylation

We identified two probes mapping to a 1.7 kbp CpG island within the *FAM50B* promoter. These probes were hypermethylated in the genome-wide mUPD (average β-values of 0.86), but hypomethylation in the three pUPD samples mUPD (β-value of 0.23, 0.39, 0.31). Allelic bisulphite sequencing showed that the methylation profile of CpG 143 differs between the 5′ and 3′ ends. The 5′ region flanking the SNP rs2239713, overlapping the *FAM50B* promoter, is a maternally methylated DMR in placenta-, leukocyte- and kidney-derived DNA, while the 3′ region near rs34635612 is fully methylated on both parental alleles. Despite this methylation gradient, the *FAM50B* gene is paternally expressed in placenta ($n = 6$) (Fig. 2).

The absence of allelic methylation at the mouse orthologues of *ZNF597*, *NAT15* and *FAM50B* is associated with biallelic expression

To determine whether the allelic expression of the novel imprinted transcripts was conserved in mouse, we investigated the allele-specific expression using RT–PCR amplification across transcribed SNPs. Mouse tissues were derived from interspecies crosses at both embryonic day E9.5 and post-natal day 1. The *Fam50b* gene has two isoforms with alternative first exons. We could only detect expression in testis, which was derived from both parental alleles. Exon 2 of Fam50b corresponds to an X-chromosome-derived retrogene and overlaps a methylated CpG island.

The *Nat15* and *Znf597* genes share two different promoter CpG islands, CpG 35 and CpG 87 that are orthologous to the *ZNF597* DMR and the *NAT15* isoform 2 promoters, respectively. In mouse, both of these regions are unmethylated. Both *Nat15* isoforms are predominantly expressed in
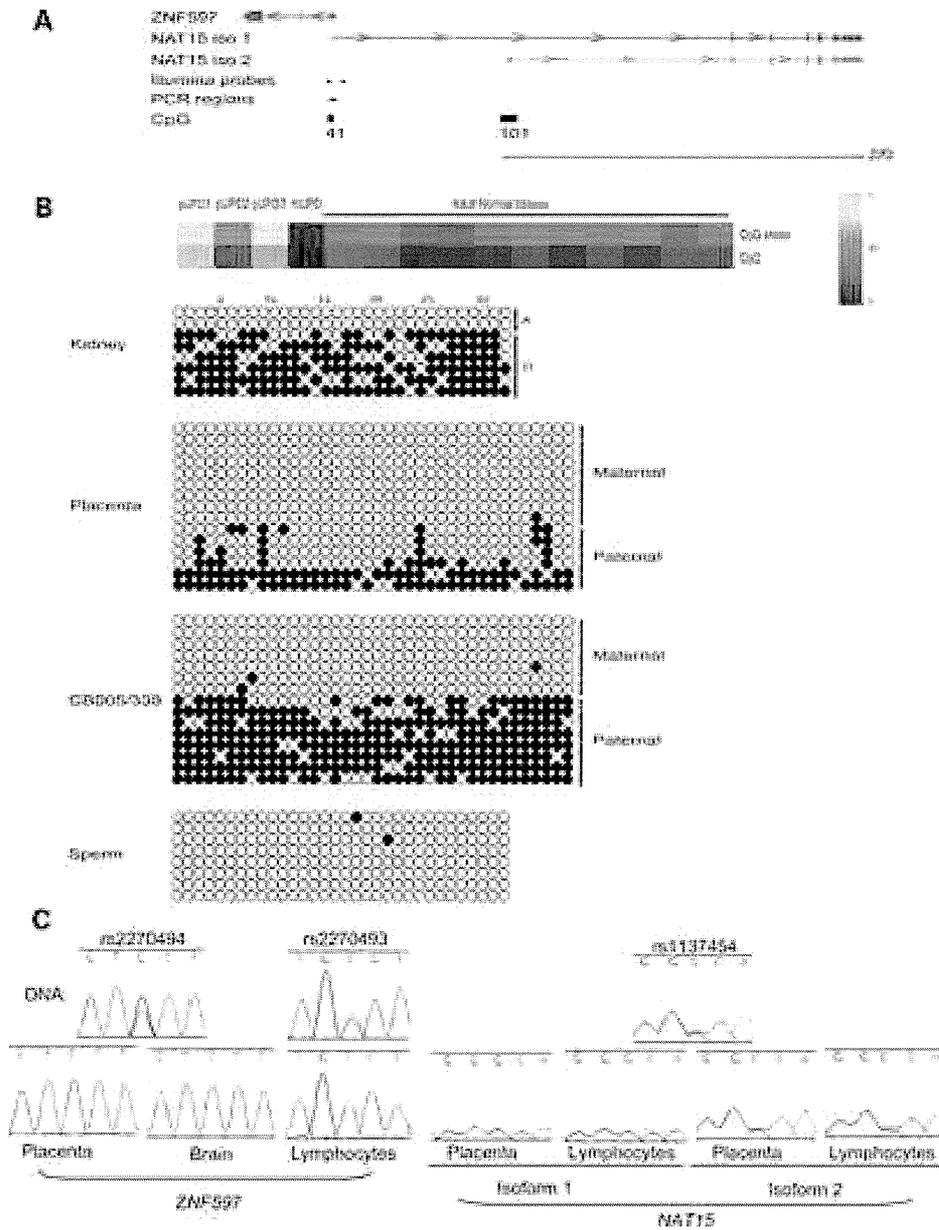
Figure 1. (**A**) Map of the *ZNF597-NAT15* locus on human chromosome 16, showing the location of the various transcripts, CpG islands, Illumina probes and bisulphite PCR regions (red transcripts are maternally expressed, blue paternally expressed and grey are expressed from both parental alleles. Arrows represent the direction of transcription) (not drawn to scale). (**B**) Heat map of the Infinium HumanMethylation27 BeadChIP for the *ZNF597* CpG probes (cg24333473 in CpG island; cg14654875 in CpG shore), with confirmation of allelic methylation in kidney, placenta and cord blood derived DNA. Each circle represents a single CpG dinucleotide and the strand, a methylated cytosine (filled circle) or an unmethylated cytosine (open circle). The same region was analyzed in sperm-derived DNA. (**C**) The sequence traces show allelic expression for the *ZNF597* and *NAT15* genes.

brain and testis, which is equally derived from both parental alleles. The variants of *Znf597* were expressed in E9.5 whole embryo, yolk sac and placenta, and in individual tissues later in development. Allelic expression analysis revealed that these transcripts were not imprinted, with equal expression from both parental chromosomes (Fig. 3).
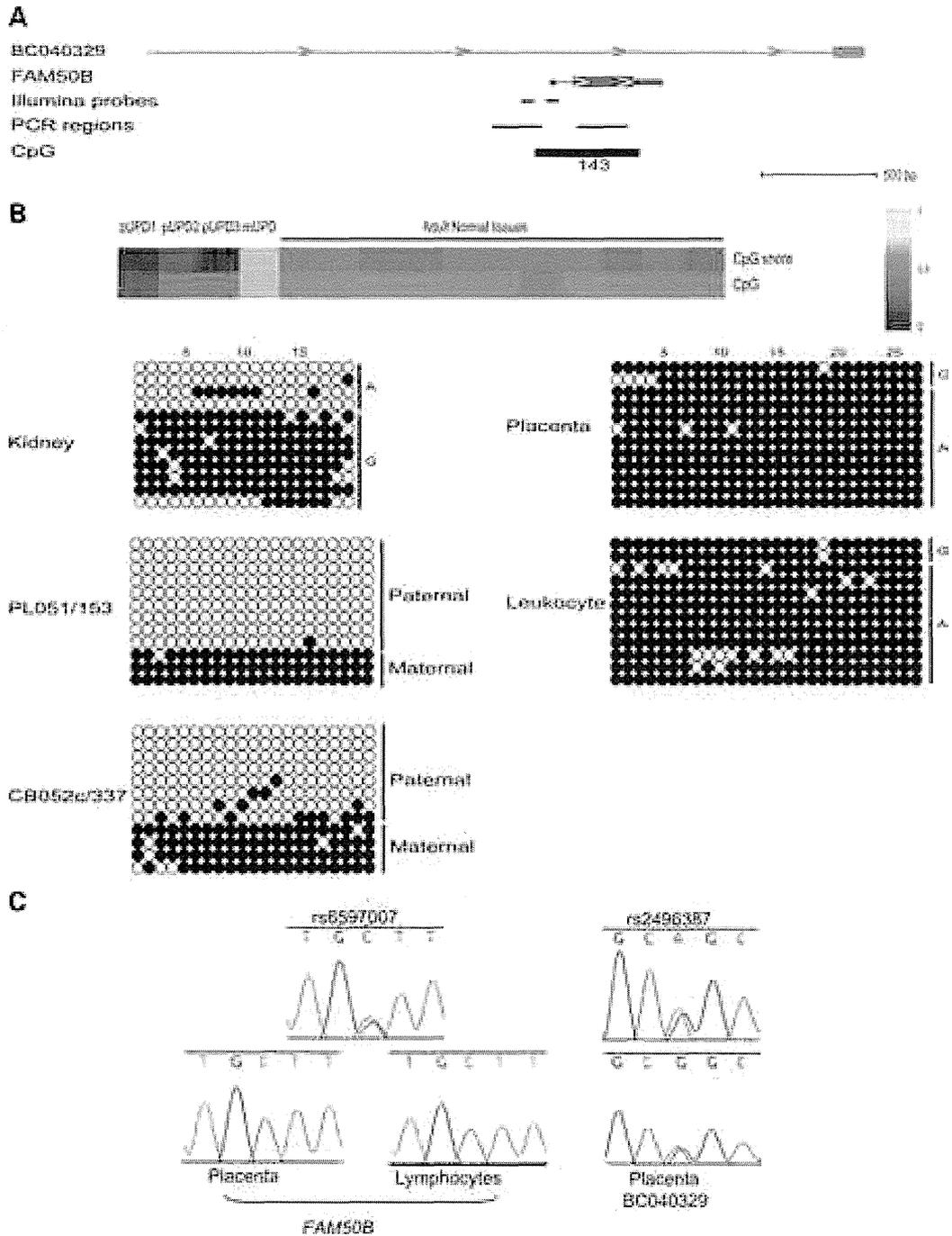
Figure 2. (A) A map of the *FAM50B/BC040329* locus, with the location of the of the CpG island (not to scale). (B) Heat map for CpG probes mapping to the *FAM50B* promoter (cg01570885; cg03202897) and the subsequent analysis of allelic methylation in various tissues. The methylation profiles on the left are from the 5′ CpG island region, while those on the right are from the 3′ region. (C) The allelic expression of *FAM50B* and the host gene in term placenta and leukocytes.
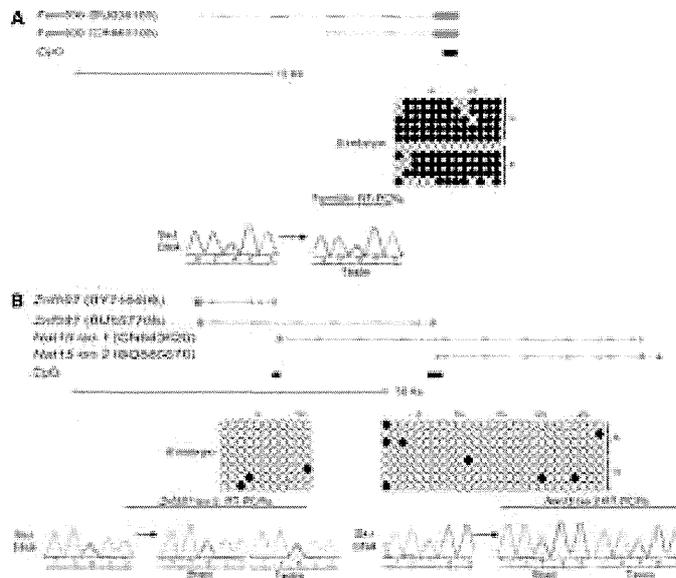
Figure 3. Schematic maps of the *Fam50b* (**A**) and *Znf597/Nat15* genes (**B**), with the location of the alternative promoter regions. The methylation status of the orthologous CpG islands associated with each domain was examined in embryo-derived DNA. The allelic expression of each gene in various mouse tissues from reciprocal mouse crosses. For clarity, only the expression in B6 × JF1 tissues is shown.

DISCUSSION

Identification of new human imprinted genes requires screening human samples

Most screens for new imprinted genes are undertaken in mouse with subsequent confirmation of the imprinting status of the human orthologues. Despite the success, this approach will not identify imprinted loci specifically imprinted in humans. To date, very few imprinted genes are human-specific, however, these rare transcripts do exist as highlighted by the paternally expressed *L3MBTL*, *C19MC* and *RB1* genes (22,29,30). Using DNA from Beckwith–Wiedemann and Silver–Russell-like phenotypes with reciprocal genome-wide UPDs, we have performed a comprehensive screen of ~0.1% of the human methylome. Despite the extensive coverage of Illumina Infinium Human Methylation27 BeadChip microarray, we identified very few novel imprinted loci. However, it must be noted that paternal germline DMRs are not associated with CpG islands, and therefore maybe remote from gene promoters and promoter CpG islands present on the array.

The predicted number of imprinted genes varies with estimates from 200–2000 transcripts in mouse, with one transcriptome-wide analysis, using the ultra sensitive RNA-seq technology, identifying over 1000 transcripts in brain with parent-of-origin expression bias (31). Recent studies have predicted and experimentally verified imprinted genes based on sequence and epigenetic characteristics. For example, human imprinted regions significantly lack short interspersed transposable elements in comparison with the rest of the genome and are associated with CpG islands (32,33). Using a bioinformatics approach, Luedi *et al.* (34) predicted 156 imprinted genes in humans based on similarity

with known imprinted transcripts, confirming the maternal expression of *KCNK9*. In addition, the paternally expressed *MCTS2* gene was identified through a hypothesis-driven search for intronic X-chromosome-derived retrotransposons that are associated with CpG island promoters (35). Interestingly, *FAM50B* is also an imprinted X-chromosome-derived retrogene gene and was correctly identified by Luedi *et al.* (34) during their computational screening and the imprinting status recently confirmed (36).

We wished to identify additional imprinted loci based on data generated in previously published analyses. We have compared our hemimethylated data set against the 156 bio-informatically predicted imprinted genes and the 82 candidates predicted due to unequal representation of alleles in public EST libraries and expression genotype arrays (37,38). We found that fifteen out of one hundred and fifty-six and nine out of eighty-two, respectively, were present in our data set. However, none of these additional genes had a methylation profile consistent with an imprinted DMR, highlighting the high false-positive rates of bioinformatic predictions (Supplementary Material, Fig. S4). From our observations, we predict that the majority of human DMRs overlapping promoters have been identified. Following analysis of more than 14 000 genes, we identified only two new imprinted DMRs. Extrapolating this trend to the 34 702 annotated RefSeq genes, we predict that there will be around five additional unidentified DMRs in the human genome, resulting in a total of ~35.

Parent-of-origin DNA methylation is not the only epigenetic signature associated with imprinted DMRs (reviewed in 9). Recently, a chromatin signature has been shown to mark imprinted DMRs; with trimethylation of lysine 9 of histone

H3 (H3K9me3) and trimethylation of lysine 20 of histone H4 (H4K20me3) associated with the DNA methylated allele (39), while the unmethylated allele is enriched for the transcription-ally permissive Lysine 4 methylation of histone H3 (H3K4me2/3) (40). The combination of differential DNA methylation between sperm and somatic tissues and an over-lapping H3K9me3 and H3K4me3 signature has recently been used to identify 11 new candidate DMRs in mouse (41). With the availability of human ChIP-seq derived genome-wide data sets for most histone modifications (42,43), it would be interesting to determine if this histone signature recognized in mouse can be used to identify novel human imprinted DMRs. Interrogation of the NHLBI ChIP-seq data set (http://dir.nhlbi.nih.gov/papers/lmi/ep igenomes/hgtcell.aspx) revealed that the *RB1*, *ZNF597* and *FAM50B* DMRs are enriched for both H3K4me3 and H3K9me3, with the later two regions harboring functional CTCF binding sites (data not shown).

The regulation of imprinted domains on human chromosomes 13 and 16

The *RB1* DMR has previously been proposed to contain the promoter of the paternally expressed *E2B-RB1* isoform (22). We were unable to identify coding SNPs within the *RB1* gene that would allow us to determine the allelic expression in our cohort of tissues. However, we were able to show that the *LPAR6* gene, encoding lysophosphatidic acid receptor 6 located in intron 16 of *RB1* is biallelically expressed, suggesting that the *RB1* DMR does not influence the expression of this gene (Supplementary Material, Fig. S3).

The maternal expression of *ZNF597* has previously been shown in leukocytes (28). Here, we show that the *ZNF597* DMR acts as a bidirectional silencer, which orchestrates the paternal silencing of *ZNF597* and *NAT15*. This organization is reminiscent of *PEG10-SGCE* domain on human 7p22 (44). We did not observe methylation in DNA isolated from mature sperm, which suggests that this region acquires methylation during early somatic development (Fig. 1). All known somatic DMR are associated with nearby germline DMRs, which regulate the methylation in a hierarchical fashion (23,45,46), implying a yet to be identified germline DMR is situated within the vicinity of the *ZNF597* gene.

The maternally expressed *NAT15* is a highly conserved protein coding gene with two alternative first exons, with only isoform I subject to imprinting. In addition, there is evidence from EST libraries of an ncRNA (genbank: DA387972) that originates from the *NAT15* isoform 1 promoter and con-tinues past the exon–intron splice site to produce a ~550 bp transcript. Unfortunately, we were unable to detect expression of this transcript in our tissue set, so we could not determine if this ncRNA is imprinted.

FAM50B is an imprinted retrogene

Sequence analysis revealed that the *FAM50B* transcript (pre-viously named *X5L*) is a retrotransposon that originated from *FAM50A/XAP5* within Xq28. Unlike other classical retro-genes, this gene has an intron in the 5′ UTR in both humans and mouse, which has no counterpart in its parental gene. It

is likely that the intron was inserted after retroposition, poss-ibly during recruitment of a functional promoter region (47). Interestingly, several other imprinted genes have been shown to originate from retrotransposition from the X-chromosome genes (35,48). *FAM50B* is ubiquitously expressed, and is inserted within the intron of a host transcript *BC040329*, which is predominantly expressed in testis with low detection in brain and placenta (data not shown). This host gene is bial-lelically expressed in placenta ($n = 7$) (Fig. 2), of which two samples exhibited imprinted expression of *FAM50B*.

Discrepancy between imprinted DMR methylation screens

The quantitative methylation values obtained using the Illumina Infinium platform makes it suitable for comparing reference and test samples. This approach has previously been used to screen for imprinted DMRs using paternally derived androgenetic complete hydatidiform moles versus maternally derived mature cystic ovarian teratomas and in patients with maternal hypomethylation syndrome (24,49). In both cases, the genetic material analyzed is not ideally suited for comprehensive screening for novel imprinted loci. This is because it is currently unknown to what extent the DNA methylation profile is altered in ovarian teratomas, and any differences maybe due to the uniparental nature of the sample or tumorgenic changes, and candidates obtained from comparisons with complete hydatidiform moles may simply reflect tissue-specific differences. This is highlighted by the fact that of the 95 candidate probes identified by Choufani et al. (49), 68 overlapped with our hemimethylated data set (Supplementary Material, Fig. S4) with only *ZNF597* DMR being identified in both screens. These authors also suggest that *AXL*-promoter region is a DMR, but this was not ident-ified using our genome-wide UPDs, and bisulphite PCR and sequencing of our samples revealed a non-allelic mosaic methylation profile (Supplementary Material, Fig. S5). In addition, the methylation profiles obtained from comparing normal and maternal hypomethylation samples will only facilitate the identification of a subset of imprinted DMRs, since *ZFP57* mutations do not effect the maintenance of all maternally methylated imprinted DMRs equally (50,51).

Functional relevance of the new imprinted domains

Very little is known about the role of *FAM50B*, *ZNF597* and *NAT15*, with no previous publications describing functional studies. The three new imprinted regions we identify all map to chromosomes for which recurrent chromosomal UPDs have been reported. With the exception of pUPD and the over-expression of *PLAGL1/HYMAI* in Transient Neonatal Diabetes Mellitus, the UPDs for these chromosomes are not associated with obvious developmental phenotypes and most cases were identified because of the unmasking of mutant recessive alleles (reviewed in 52,53).

CONCLUSIONS

Our study has assisted in defining a comprehensive catalog of human imprinted genes. The use of extremely rare reciprocal

genome-wide UPD samples in unbiased methylation screens such as bisulphite genome sequencing will aid the identification of additional imprinted loci, which will facilitate study of genetic diseases associated with aberrant imprinting. The general trend until now has been that, while imprinted genes play an important role in fetal development and behavior, evolutionary forces dictated by the genetic conflict have allowed for a lack of conserved imprinting between mouse and humans (54). However, our screen has identified new human-specific imprinted transcripts, all of which have conserved gene orthologues in many taxa. These genes have selected imprinting as a mechanism of transcriptional regulation in humans despite the risk of being functional hemizygous.

MATERIALS AND METHODS

The human reciprocal genome-wide UPD samples

Genomic DNA isolated from three previously described Beckwith–Weidemann syndrome-like cases (16–18) and one Silver–Russell syndrome-like patient (19) was used in this study. Each of these cases had undergone extensive molecular characterization to confirm genome-wide UPD status and level of mosaicism. We used DNA isolated from leukocytes as these samples had minimal mosaicism of a biparental cell line. The genome-wide BWS samples had 9, 11 and 15% biparental contribution, whereas the genome-wide SRS sample had 16%.

Human tissues

Two independent tissue collections were used in this study. All tissues were collected after obtaining informed consent. The Spanish collection was from the Hospital St Joan De Deu tissue cohort (Barcelona, Spain). Normal peripheral blood was collected from adult volunteers aged between 19 and 60 years old. A selection of normal brain samples was obtained from BrainNet Europe/Barcelona Brain Bank. The Japanese tissues were collected at the National Center for Child Health and Development (Tokyo, Japan) and at the Saga University Hospital.

DNA was extracted using either the standard phenol/chloroform extraction method or the QIAamp DNA Blood Midi Kit (Qiagen). RNA was extracted using either Trizol (Invitrogen) or Sepasol®-RNA I Super G (Nacalai Tesque) and cDNA synthesis was carried out as previously described (54). Ethical approval for this study was granted by the Institutional Review Boards at the National Center for Child Health and Development and Saga University and Hospital St Joan De Deu Ethics Committee (Study number 35/07) and IDIBELL (PR006/08).

Cell lines and mouse crosses

Wild-type mouse embryos and placentas were produced by crossing C57BL/6 with *Mus musculus molosinus* (JF1) mice. C57BL/6 (B6) mice were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and JF1/Ms (JF1) mice were obtained from the Genetics Strains Research Center at the National Institute of Genetics, Japan. All animal husbandry and breeding was approved and licensed by the National Research Institute for Child Health and Development, Japan (Approved number A2010–002).

Illumina Infinium methylation27 BeadChip microarray analysis

Approximately 1 μg DNA from the reciprocal genome-wide UPDs, placenta, leukocytes, brain, muscle, fat, buccal cells was subjected to sodium bisulphite treatment and purified using the EZ GOLD methylation kit (ZYMO, Orange, CA, USA). This DNA was then hybridized to the Illumna Infinium Human Methylation27 BeadChip microarray either at the Centro Nacional de Investigaciones Oncologicas (Madrid, Spain) or Genome Science Division, Research Center for Advanced Science and Technology (University of Tokyo, Japan) using Illumina-supplied reagents and protocols. The loci included on this array and the technologies behind the platform have been described previously (55). Before analyzing the methylation data, we excluded possible sources of technical biases that could alter the results. We discarded 109 probes because they had a false-positive rate >0.1. We also excluded 261 probes because of the lack of signal in one of the 11 DNA samples analyzed. Lastly, prior to screening for novel imprinted DMRs, we excluded all X chromosome CpG sites. Therefore, in total we analyzed 26 152 probes in all DNA samples. All hierarchical clustering and β-value evaluation was performed using the Cluster Analysis tool of the BeadStudio software (version 3).

Allelic methylation analysis

A panel of placenta-, leukocyte-, brain- and kidney-derived DNAs were genotyped to identify heterozygous samples. These DNA were converted using the EZ GOLD methylation kit. Approximately 100 ng of converted DNA was used for each bisulphite PCR. Bisulphite-specific primers (Supplementary Material, Table S1) which incorporate the SNPs were used with Hotstar Taq polymerase (Qiagen, West Sussex, UK). Amplifications were performed using either 45 cycles or a nested PCR using 35 cycles for each round. The subsequent PCR products were cloned into pGEM-T Easy vector (Promega) for subsequent sequencing.

Allelic expression analysis

Genotypes on DNA were obtained for exonic SNPs identified in the UCSC browser (NCBI36/hg18, Assembly 2006) by PCR. Sequences were interrogated using Sequencher v4.6 (Gene Codes Corporation, MI) to distinguish informative heterozygote samples. Informative samples were analyzed by RT–PCR. All primers, with the exception of those targeting *FAM50B*, are intron-crossing and incorporated the heterozygous SNP in the resulting amplicon (Supplementary Material, Table S1). RT–PCRs were performed using cycle numbers determined to be within the exponential phase of the PCR, which varied for each gene, but was between 32 and 40 cycles.

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Congenital Corneal Staphyloma as a Complication of Kabuki Syndrome

Ryuma Tanaka,[1] Toshiki Takenouchi,[1] Keiko Uchida,[1] Takeshi Sato,[2] Hiroyuki Fukushima,[1] Hiroshi Yoshihashi,[3] Takao Takahashi,[1] Kazuo Tsubota,[4] and Kenjiro Kosaki[1,5]*

[1]Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

[2]Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Kanagawa, Japan

[3]Division of Medical Genetics, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan

[4]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan

[5]Center for Medical Genetics, Keio University School of Medicine, Tokyo, Japan

Kabuki syndrome has long been clinically defined based mainly on its characteristic eye features. The recent discovery of *MLL2* as a causative gene of Kabuki syndrome has enabled the extreme end of the phenotype to be explored. We herein report on two patients with striking visible congenital staphyloma at birth. A diagnosis of Kabuki syndrome was subsequently made in both patients based on a constellation of characteristic eye features, cardiac abnormalities and severe developmental delay, and finally by the confirmation of *MLL2* mutations. In conclusion, congenital corneal staphyloma is a complication of Kabuki syndrome with *MLL2* mutations. © 2012 Wiley Periodicals, Inc.

Key words: Kabuki syndrome; staphyloma; CHARGE syndrome

on these unusual features. As an example of an extreme phenotype, we herein present two *MLL2* mutation-positive patients who had congenital corneal staphyloma, a severe form of corneal defect that causes a forward protrusion between the eyelids [Leff et al., 1986; Nelson and Olitsky, 2005].

INTRODUCTION

Kabuki syndrome is a congenital syndrome with characteristic facial features reminiscent of the make-up patterns used by Japanese Kabuki actors. Since Kuroki and Niikawa independently described this entity for the first time in 1981 [Kuroki et al., 1981; Niikawa et al., 1981], Kabuki syndrome has become a clinically defined entity, with its diagnosis based mainly on its characteristic facial gestalt. Although Kabuki syndrome was long suspected of being a genetically defined entity, its exact genetic basis remained unknown until 29 years after its initial description, when exome sequencing identified the majority of Kabuki syndrome patients to be caused by mutations in the *MLL2* gene on chromosome 12q12-q14 [Ng et al., 2010]. Now that the genetic basis of Kabuki syndrome has been defined, its phenotypic spectrum should be explored.

Although various ophthalmologic abnormalities other than the typical Kabuki appearance, such as coloboma, had been described in Kabuki syndrome prior to the aforementioned discovery of *MLL2* gene mutations in Kabuki syndrome [Ming et al., 2003], the extreme ends of the ophthalmologic phenotype have not yet been labeled because of the uncertainty of clinical diagnoses based

CLINICAL REPORT

Patient 1

The propositus was a Japanese boy who was prenatally diagnosed as having left pyelectasis and was born at 39 and 4/7 weeks of gestation via vacuum-assisted spontaneous vaginal delivery to a 27-year-old primiparous mother. His birth weight was 3,614 g, and his Apgar scores were 7, 7, and 8 at 1, 5, and 10 min, respectively. At birth, he was noted to have a small but visible structure protruding out of his right eye (Fig. 1). The proband had low-set ears, a high arched
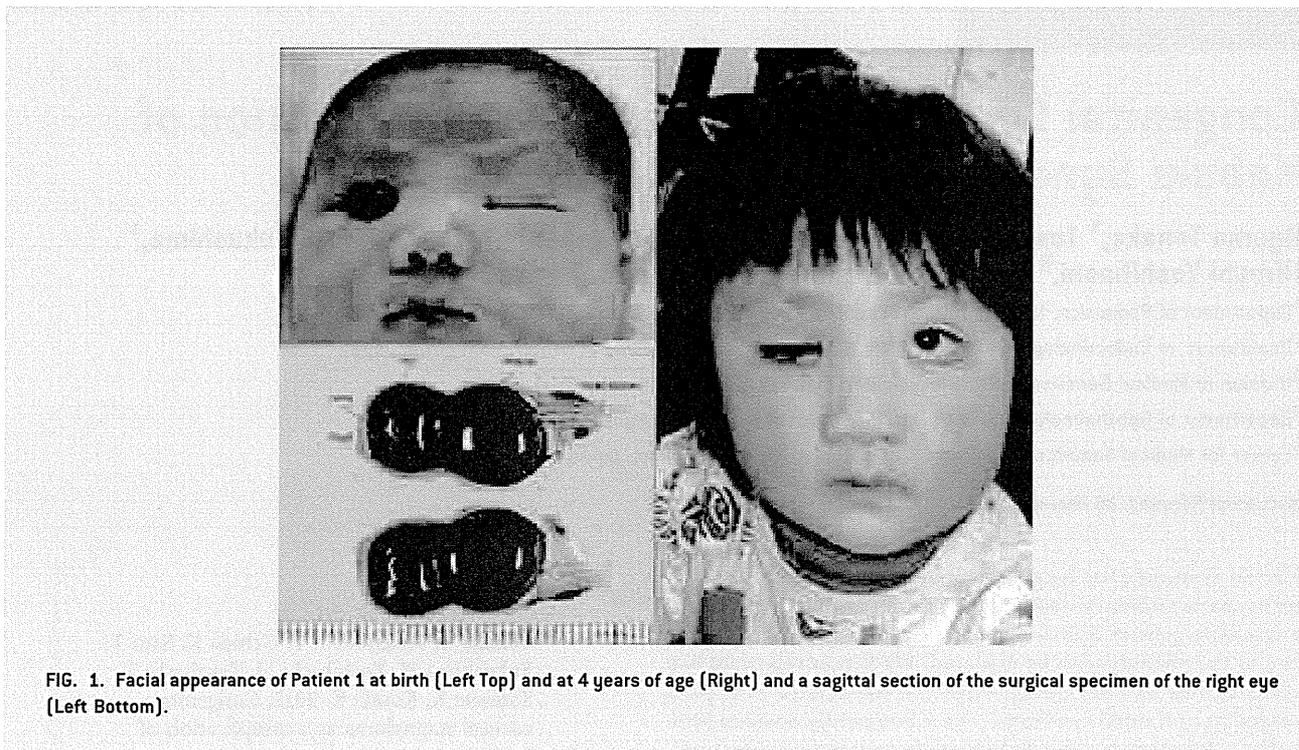
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FIG. 1. Facial appearance of Patient 1 at birth (Left Top) and at 4 years of age (Right) and a sagittal section of the surgical specimen of the right eye (Left Bottom).

palate, and a sacral dimple. In addition to a small ventricular septal defect, he was noted to have a left double-renal pelvis, hydronephrosis, ureteropelvic junction stenosis, and right renal malrotation, all of which were managed medically. At 1 month of age, he underwent the total enucleation of his right eye. The pathology report indicated keratoectasia, without evidence of malignancy. His left eye was normal. At age 4 years, he began to ambulate without support but had no meaningful words. A mutation analysis of *MLL2* revealed a truncating mutation in exon 49 (c.15844C>T, p.R5282X).

Patient 2

The second propositus was a non-consanguineous Japanese boy who was born at 39 and 3/7 weeks of gestation via normal spontaneous vaginal delivery. There was no family history of any known inherited condition. His birth weight was 3,379 g, and his height was 50 cm. At birth, bilateral microphthalmia with a right congenital corneal staphyloma were noted (Fig. 2). He underwent a total enucleation of the right eye at the age of 1 month. He had ventricular and atrial septal defects accompanied by symptomatic heart failure, which was treated medically. He has been treated with phenobarbital for epilepsy since the age of 11 months. His motor and mental development were significantly delayed. He first sat at the age of 2 years and 5 months, and he walked independently at the age of 9 years. A clinical diagnosis of Kabuki syndrome was made at the age of 27. A mutation analysis revealed a de novo missense mutation in exon 51 of the *MLL2* gene (c.16294C>T, p.R5432W),

confirming the diagnosis of Kabuki syndrome. Currently, he is 30 years old and is unable to communicate verbally; he requires assistance with all his daily activities.

DISCUSSION

Here, we report on two patients with Kabuki syndrome and *MLL2* mutations who had visible congenital corneal staphyloma at birth.
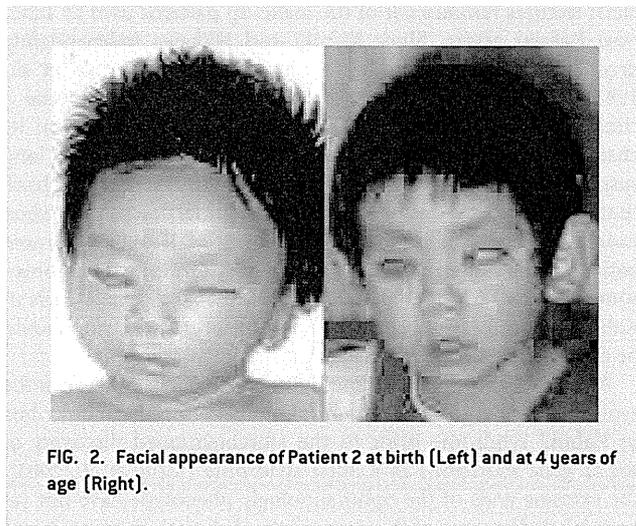

FIG. 2. Facial appearance of Patient 2 at birth (Left) and at 4 years of age (Right).