European Journal of Human Genetics (2008) 16, 453–461

www.nature.com/ejhg

ARTICLE

Clinical characterisation of the multiple maternal hypomethylation syndrome in siblings

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We present the first clinical report of sibs with the multiple maternal hypomethylation syndrome. Both sisters presented with transient neonatal diabetes mellitus (TNDM). By methylation-specific PCR of bisulphite-treated DNA, we found a mosaic spectrum of hypomethylation at the following maternally methylated loci in both sibs: *ZAC* (6q24), *KCNQ1OT1* (11p15.5), *GRB10* (7p11.2–12), *PEG3* (19q13), *PEG1/MEST* (7q32), and *NESPAS* (20q13). While the older sister has a milder phenotype, the younger one was severely ill and died at 11 months of age. Despite phenotypic differences, the sisters had several manifestations of both TNDM and BWS in common. The family is highly consanguineous, and the parents are first cousins. We suggest that the genetic defect in this family is a novel, most likely autosomal recessive defect of methylation mechanisms, either in the sisters or in their mother, affecting her oocyte imprinting. The recurrence with affected sibs as reported in this family has implications for genetic counselling.
European Journal of Human Genetics (2008) **16,** 453–461; doi:10.1038/sj.ejhg.5201993; published online 16 January 2008

Keywords: genomic imprinting; DNA methylation; Beckwith–Wiedemann syndrome; transient neonatal diabetes mellitus; multiple maternal hypomethylation syndrome

Introduction

Methylation of CpG dinucleotides in DNA is one of the epigenetic imprinting mechanisms through which alleles are differentially expressed, depending on their parental origin. We previously documented that aberrant methylation in imprinting disorders may affect more than one single locus: we found concurrent loss of methylation (LOM) of the imprinted transient neonatal diabetes mellitus (TNDM; MIM 601410) differentially methylated region (DMR) at 6q24 and Beckwith–Wiedemann syndrome (BWS; MIM 130650) DMR2 (*KCNQ1OT1)* at 11p15.5 in two TNDM patients.[1] Similar dual LOM had previously been demonstrated in two patients by Arima *et al*.[2] Subsequently, we showed that 6 out of 12 anonymised TNDM patients with LOM at TNDM DMR (6q24) in addition had a diverse spectrum of maternal hypomethylation at multiple imprinted loci including *KCNQ1OT1* (11p15.5), and we suggested the existence of a maternal hypomethylation syndrome.[3] A similar spectrum of multiple maternal hypomethylation was found by Rossignol *et al*,[4] in 10 out of 40 LOM BWS patients.

With informed consent, we tested the methylation status at multiple imprinted loci in one of our two initial patients

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Received 17 August 2007; revised 15 November 2007; accepted 30 November 2007; published online 16 January 2008

reported by Mackay *et al*[1] and her younger sister who also had TNDM. We here describe their family history, clinical phenotypes, development, and laboratory results.

Methods

Clinical investigations

The family history was recorded from the mother, who gave her written informed consent. The medical records were reviewed in detail, and both girls were re-examined as part of the study.

Laboratory investigations

Methylation-specific PCR and pyrosequencing of bisulphite-induced polymorphisms Methylation ratiometry of DNA from the sisters and their parents was performed for the loci *ZAC* (6q24), *GRB10* (7p11.2–12), *PEG1/MEST* (7q32), *KCNQ1OT1* and *H19* (both 11p15.5), *DLK1* (14q32), *SNRPN* (15q11–12), *PEG3* (19q13), and *NESPAS* (20q13) by methylation-specific PCR (MS-PCR) following bisulphite treatment, as previously described.[1,3,5] Primer sequences for *NESPAS* were derived from Williamson *et al*.[6] Aberrant results were confirmed by either pyrosequencing[7] or MS-PCR with another primer set. Control data were drawn from previous publications,[1,3] with supplementary MS-PCR at some loci to obtain an epigenotype of all 120 anonymised normal controls at all the loci. For the sisters, the MS-PCR was performed on genomic DNA extracted from EDTA-stabilised whole blood, cells from repeated buccal swabs, and skin fibroblasts.

Southern blot analysis At the *KCNQ1OT1* locus, the results of MS-PCR were also confirmed by Southern blot analysis with the *LIT1* probe (AA155639) after digestion of genomic DNA with *Bam*HI and *Not*I.[8]

Test for uniparental disomy by polymorphic microsatellite markers Analysis of genomic DNA from the sisters and parents with the following polymorphic microsatellite markers was carried out to test for uniparental disomy: D6S292, D6S314, D6S1704 (6q23–24), D11S TH, D11S1318, D11S4088, and D11S4146 (11p15.4–15.5) (http://genome.ucsc.edu/).

Methylation-sensitive multiple ligation-dependent probe amplification analysis Genomic and methylation-sensitive MLPA analysis of 11p15.5, using SALSA MS-MLPA kit ME030 (MRC Holland), was performed on leukocyte-derived DNA, to test for deletion or duplication of genes on 11p15.5, and to confirm the hypomethylation of *KCNQ1OT1* in the sisters.

Karyotyping and screening for heterochromatin instability Chromosome analysis of PHA-stimulated lymphocytes from the sisters and their parents was performed after G- and Q-banding (standard protocols; 450–500 banding level). One hundred metaphases from the younger sister were screened for micronuclei and paracentromeric heterochromatin instability, particularly of chromosomes 1 and 16, to exclude the immunodeficiency, centromeric instability, and facial anomalies syndrome (ICF syndrome; MIM 242860).

Subtelomeric MLPA analysis To rule out a cryptic unbalanced translocation in the sisters, subtelomeric MLPA analysis was carried out with the SALSA MLPA kits P036B and P070 (MRC Holland).

Sequencing for* KCNJ11 *mutations DNA from the older sister was sequenced for mutations in *KCNJ11* by Molecular Genetics Laboratory, Royal Devon and Exeter NHS Healthcare Trust (Exeter, UK).

Screening for homozygosity at candidate gene loci by single-nucleotide polymorphisms (SNPs) and polymorphic microsatellites, and sequencing of the* NLRP7 *gene By GeneChip® Mapping 10K 2.0. SNP Array (Affymetrix) and flanking microsatellite markers, genomic DNA from the sisters and their parents was analysed to screen for homozygosity at the following candidate gene loci: *DNMT1* (19p13.3–13.2), *DNMT2* (10p15.1), *DNMT3A* (2p23), *DNMT3B* (20q11.2), *DNMT3L* (21q22.3), and *NLRP7* (19q13.4). Exons and intron–exon boundaries of *NLRP7* in the mother were sequenced using the Golden Path sequence (http://genome.ucsc.edu/) and primers designed by ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html).

Results

Clinical investigations

The sisters were the only children born to healthy consanguineous Turkish parents (Figure 1). The parents, maternal, and paternal grandparents are all first cousin couples. Five relatives have had either type 1 or 2 diabetes mellitus, or gestational diabetes. Several children in the family have died as neonates or in infancy for unknown reasons in Turkey. No relatives are known to have TNDM, BWS, or other imprinting disorders. A male cousin of the sisters suffers from epilepsy and psychomotor retardation of unknown cause.

The older sister[1] has been followed up to the age of 5 years and 7 months (Figure 2); the younger sister until her death at 11 months of age. Their phenotypic features and developmental details are listed in Table 1.

Neonatally, the combination of hyperglycaemia, macroglossia, and intrauterine growth retardation in the older sister led to the clinical suspicion of TNDM. This diagnosis was subsequently confirmed by the finding of LOM at 6q24. At 7 months, the diabetes went into remission, but relapsed at the age of 2 years and 8 months. At 4 years and 6 months of age, insulin was successfully substituted with an oral sulphonyl urea, which is still her medication

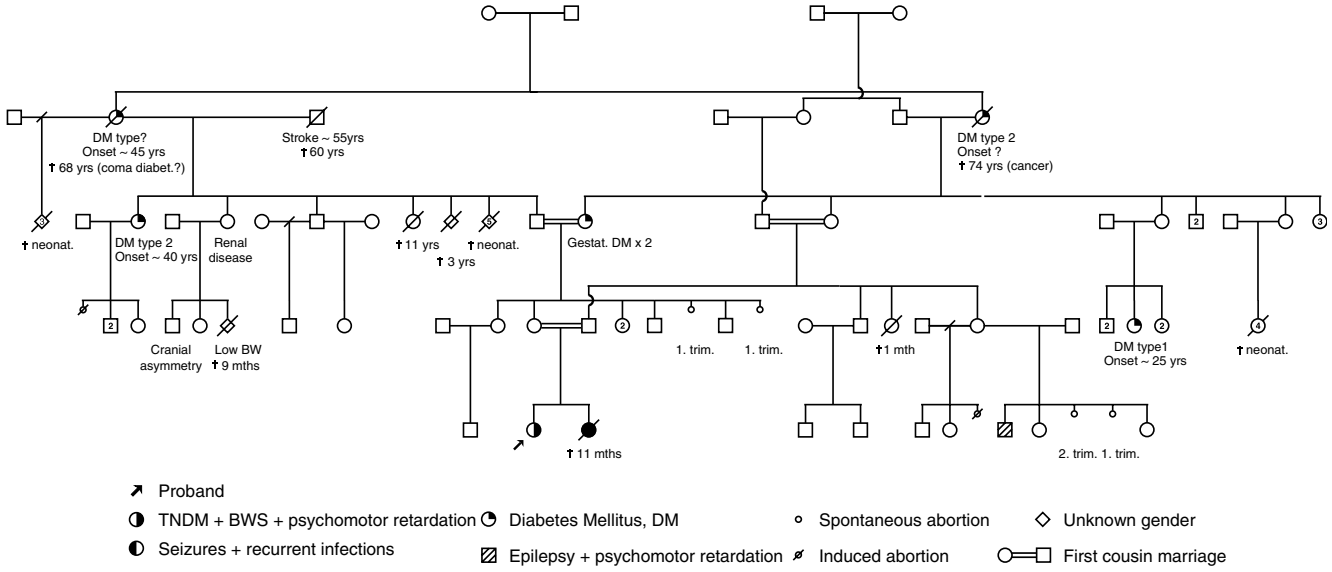

Figure 1 Pedigree of the two sisters with multiple maternal hypomethylation and their family. The causes of death are indicated, where available.

Figure 2 Older sister, 5 years and 7 months of age.

(tolbutamide, 500 mg three times a day). Her postnatal growth, in contrast to prenatal growth, is significantly accelerated, and her psychomotor development is mildly retarded.

The younger sister also presented with neonatal diabetes and, initially, seemed to be relatively mildly affected. From about 3 months of age, however, she had increasing failure to thrive and was repeatedly hospitalised due to recurrent infections. Clinically, she was suspected of an immunodeficiency, but immunoglobulins and Mannan-binding lectin were normal. She had severe growth retardation with no postnatal catch-up, and gross psychomotor retardation. She required insulin until she died.

A comparison between the clinical features of the sisters, and the features of TNDM and BWS is shown in Table 2.

Laboratory investigations

The results of the MS-PCR are shown in Table 3, and examples of the methylation analyses appear in Figure 3.

Both sisters had hypomethylation at multiple imprinted, maternally methylated loci. The hypomethylation was mosaic with respect to the cell types affected and level of methylation. At TNDM DMR (6q24) and *GRB10* (7p11.2–12), both sisters had complete LOM in all cell types analysed, while there was a spectrum of hypomethylation at *KCNQ1OT1* (11p15.5), *PEG3* (19q13), *PEG1/MEST* (7q32), and *NESPAS* (the younger sister had only minimal alteration in methylation at *NESPAS*). *SNRPN* (15q11–12), *H19* (BWS DMR1; 11p15.5), and the *DLK1* DMR (14q32) showed normal methylation. No hypomethylation was detected in any control samples. The parents had normal methylation at all loci.

The results of methylation-sensitive multiple ligation-dependent probe amplification (MS-MLPA) analysis of 11p15.5 on blood-derived DNA indicated a moderate mosaic hypomethylation at *KCNQ1OT1* in the older sister, confirming the results of MS-PCR and Southern blot (data not shown). For the younger sister, the result of MS-MLPA at this locus was within normal limits, which is in accordance with our finding that subtle methylation abnormalities may go undetected by this method (personal

Table 1 Clinical phenotype and development of the sisters

	Older sister[a]	*Younger sister*
Conception	Natural	Natural
Maternal age/ paternal age	18 years/25 years	22 years/29 years
Amniocentesis	46, XX	Not done
Pregnancy	Abnormal ultrasound findings and IUGR (see below); otherwise uncomplicated	Uneventful; nuchal translucency and malformation scan normal; only limited information available
Delivery	Uncomplicated, spontaneous; GA 40+3	Induced due to IUGR; uncomplicated; GA 39+6
Birth weight	2430 g (−2.5 SD)	2000 g (< −3 SD)
Birth length	49 cm (−1 SD)	45 cm (−3 SD)
OFC at birth	30 cm (−3 SD)	32 cm (−1 SD)
Apgar score	10/1, 10/5	8/1, 10/5, 10/10
Amniotic fluid/ placenta/ umbilical cord	Oligohydramnios/650 g/three vessels	Normal/400 g/three vessels
Phenotype	Hernia of the cord[b] (persistent omphaloenteric duct: 5 × 3 cm); bilateral postaxial polydactyly of the hands;[b] macroglossia;[b] small ductus arteriosus persistens; symmetric body, limbs, and facies; no hemihypertrophy; no brachydactyly observed clinically; first tooth eruption at 8 months	Macroglossia; umbilical hernia (observed at $2\frac{1}{2}$ months); bilateral ear lobe creases (observed at 5 months); tracheomalacia; no hemihypertrophy; hypertrophic cardiomyopathy; small atrial septal secundum defect; cerebral MRI ($6\frac{1}{2}$ months): severe hypoplasia of the corpus callosum, absence of occipital horn of the left ventricle, decreased apparent diffusion coefficient of the capsula interna and optical tract
Onset and treatment of neonatal diabetes	Blood glucose 12.2 mmol/l (day 7); glucosuria; no ketoacidosis; insufficient β-cell function (nonfasting C-peptide <160 pmol/l); no diabetes-associated antibodies; insulin from day 15 to 7 months; Initially difficult to regulate/high doses required (~2.5 units/kg/day)	Blood glucose 9.5 mmol/l (day 1) → 14.3 mmol/l (day 7); glucosuria; no ketonuria; insulin from day 8
Subsequent diabetes and treatment	Relapse at 2 years 8 months: blood glucose 16.1 mmol/l; HbA_{1c} 10.3%; BMI 21.4 ~obesity; at $4\frac{1}{2}$ years, substitution of insulin with sulphonylurea (SU) because of significant β-cell function; mixed meal tolerance test (90 min Boost test) at 5 years 7 months: fasting C-peptide 550 pmol/l; stimulated C-peptide 930 pmol/l; after 3 days of SU omission: fasting C-peptide 270 pmol/l; stimulated C-peptide 490 pmol/l, indicating the significant effect of SU treatment	During infections, fluctuating blood glucose levels, but no ketoacidosis; HbA_{1c} at 8 months: 6.2%; insulin required till death
Somatic health	Unremarkable; not prone to infections; abdominal ultrasound ($8\frac{1}{2}$ months and 4 years 8 months): no organomegaly or tumours	Severe failure to thrive; permanent gastric feeding tube; recurrent severe respiratory infections/septicaemia (once with candida); clinical suspicion of immunodeficiency; episodes of secondary severe uraemia, cardiac decompensation, mild liver disease, and temporary hypothyroidism; severe epilepsy; EEG highly abnormal; status epilepticus several times; alternating hypertonicity and hypotonicity; MRI ($6\frac{1}{2}$ months, during uraemia): enlarged, pale kidneys, otherwise normal organs; died ~11 months old of unknown cause; no autopsy.
Laboratory results	TSH slightly reduced at $4\frac{1}{2}$ years; at 5 years 7 months: TSH slightly elevated; T3, T4, serum parathyroid hormone, calcium, phosphate normal; no vitamin D deficiency	Immunoglobulins and subclasses normal; Mannan-binding lectin normal; no further immunological investigations obtained; serum calcium level slightly elevated during infections, normal in between; TSH normal at neonatal screen
Urine metabolic screening	No specific abnormalities ($1\frac{1}{2}$ months)	No specific abnormalities (8 days, 20 days, 6 months) (alanine slightly elevated, most likely related to the blood lactate elevations following seizures and septicaemia)
Vision	Bilateral hypermetropia (+3/+4.25); astigmatism (2 years 4 months); no cataract observed (4 years 0 month)	Complete lack of vision suspected, possibly due to abnormalities in occipital cortex; ophthalmoscopy inconclusive/no major changes; VEP not performed
Hearing	No hearing loss suspected	Profound hearing loss suspected; audiological examination inconclusive due to referred noise from tracheomalacia

Table 1 (*Continued*)

	Older sister[a]	*Younger sister*
Weight	+2.5 SD → +2 SD (3 months → now)	< −3 SD (birth →)
Length	+3 SD → +2 SD (6 months → now)	< −3 SD (birth →)
OFC curve	−3 SD → +1 SD (birth → now)	−1 SD → ≪ −3 SD (birth →)
Psychomotor development	Slightly retarded; milestones: able to sit alone 8 months, stand alone 11–12 months, walk alone 21 months, speak a few words 24 months, speak language 3.5 years; attends a group for children with special needs in her day care centre	Severely retarded; development estimated by special physiotherapists at 10 months of age ~0–1 months

BMI, body mass index; EEG, electroencephalogram; GA, gestational age; IUGR, intrauterine growth retardation; MRI, magnetic resonance imaging; OFC, occipitofrontal circumference; TNDM, transient neonatal diabetes mellitus; TSH, thyroid-stimulating hormone; VEP, visual evoked potential.
[a]TNDM patient 2 in Mackay *et al.*[1] Followed till the age of 5 years and 7 months.
[b]Abnormality also observed at successive prenatal ultrasound scans (17–34 weeks of gestation).

observation). MS-MLPA showed no evidence of allele copy number change in the sisters.

Polymorphic microsatellite marker analysis showed normal, biparental inheritance, and no evidence of deletion or duplication of chromosomes 6q23–24 and 11p15.5.

The sisters and their parents all had normal karyotypes of lymphocytes, and there were no micronuclei or heterochromatin instability, indicating ICF syndrome in the younger sister.

Subtelomeric MLPA analyses were normal, thus excluding unbalanced translocations in the sisters.

No *KCNJ11* mutation was found in the older sister at sequencing.

Analysis at the *DNMT* genes and *NLRP7*, of SNPs and polymorphic microsatellites in combination showed heterozygosity in one or both sisters, and the sisters had inherited divergent parental alleles around these loci. At SNP analysis, the mother was heterozygous at all *DNMT* loci, except for *DNMT1*, where she, like her husband and daughters, was homozygous for 8.2 Mb around the gene. When analysed with four polymorphic microsatellite markers spanning 1.58 Mb around *DNMT1*, she was, however, heterozygous for three of them. We conclude that the homozygosity of SNPs around *DNMT1* was due to non-informativity. The mother was heterozygous around *NLRP7*, and sequencing her *NLRP7* revealed no mutations.

Discussion

This is the first clinical report of sibs affected with hypomethylation at multiple imprinted, maternally methylated loci. The underlying mechanism of this novel syndrome is not known. Environmental factors may play a role in the complex processes of establishing and maintaining methylation imprinting marks in both animals[9] and humans, for example, related to assisted reproductive technology.[10] In the family presented here with affected sibs born after naturally conceived pregnancies, the aetiology of the multiple methylation aberrations is likely to be genetic. The imprinted loci found to be affected in this study are all maternally methylated in normal individuals, while the paternally methylated loci had normal methylation. Furthermore, hypomethylation was mosaic with respect to its level, the cell types and tissues affected, and the loci involved, suggesting a postzygotic origin of the aberrant pattern of DNA methylation.

Phenotypic findings

Despite many differences, the clinical phenotypes of the sisters had several features in common including a presentation with intrauterine growth retardation, neonatal diabetes, macroglossia, and umbilical defects (see Table 1). Complete LOM at TNDM DMR in both sibs could explain these findings. Macroglossia and abdominal wall defect as well as the postnatal excessive growth in sib 1 could,

Table 2 Comparison of the clinical features of the sisters with the phenotypes of TNDM and BWS

	Older sister	*Younger sister*	*TNDM*[a]	*BWS*[a]
Prenatal growth retardation	+	+	+	Overgrowth
Postnatal growth retardation	Overgrowth	+++	−	Overgrowth
Preterm delivery	−	−	−	+
Polyhydramnios	Oligohydramnios	−	−	+
Large placenta	+	+	−	+
Neonatal hyperglycaemia	+	+	+	Hypoglycaemia
Failure to thrive first 6 months	−	+++	++	−
Macroglossia	+	+	+	+
Abdominal wall defect	+	+	+	+
Hemihypertrophy	−	−	−	+
Cardiomyopathy	−	+	−	(+)
Postaxial polydactyly	+	−	−	(+)
Psychomotor retardation	+	+++	−	(+)
Diabetes later in life	+	Not known	+	−

BWS, Beckwith–Wiedemann syndrome; TNDM, transient neonatal diabetes mellitus.
[a]Some features are major characteristics of the syndrome, while others are variable. Parenthese indicate occasional feature. Not all known variable features of the syndrome are included in this table.

Table 3 Results of the MS-PCR

	Older sister			*Younger sister*			*Mother*	*Father*	*120 normal controls*[a] *(mean ± SD)*
Locus	*B*	*MB*	*FIB*	*B*	*MB*	*FIB*	*B*	*B*	*B*
ZAC[b] 6q24	∞	∞	∞	∞	∞	∞	1	1	1.00 ± 0.16
KCNQ1OT1[b] 11p15.5	4	1	1	2	1	1	1	1	1.00 ± 0.17
GRB10[b] 7p11.2–12	∞	∞	∞	∞	∞	∞	1	1	1.00 ± 0.17
PEG3[b] 19q13	∞	∞	14	∞	∞	6	1	1	1.00 ± 0.22
PEG1/MEST[b] 7q32	2	2	4	2	2	1.5	1	1	1.00 ± 0.20
NESPAS[b] 20q13	6	3	5	1	1	1.5	1	1	1.00 ± 0.21
SNRPN[b] 15q11–12	1	1	ND	1	1	ND	1	1	1.00 ± 0.16
H19[c] 11p15.5	1	1	1	1	1	1	1	1	1.00 ± 0.16
DLK1[d] 14q32	1	1	ND	1	1	ND	1	1	1.00 ± 0.17

B, DNA from EDTA-stabilised whole blood; FIB, DNA from skin fibroblasts; MB, DNA from cells obtained by mouth brush/buccal swab; MS-PCR, methylation-specific PCR; ND = not done.
The numbers in the table represent unmeth/meth ratios as exemplified in Figure 3a and c.
[a]Control data were drawn from previous publications[1,3] with supplementary MS-PCR at some loci to obtain an epigenotype of all 120 anonymised normal controls at all the loci.
[b]Maternally methylated in normal individuals.
[c]Paternally methylated in normal individuals.
[d]*DLK1* is paternally expressed, but the DMR in *DLK1* analysed here is paternally methylated in normal individuals.

however, also result from the hypomethylation at *KCNQ1OT1*, as these manifestations are major features of BWS (Table 2). Postaxial polydactyly (sib 1) and cardiomyopathy (sib 2) can also occasionally occur in BWS patients.[11,12] Sib 1's relapse of diabetes at 2 years and 8 months was early, compared to previously reported TNDM cases, where the average was 14 years with an earliest reported age of 4 years prior to this report.[13] It is possible that the obesity of the patient provoked an insulin resistance and thus an early relapse of the diabetes. We do not know whether the diabetes of the younger sister was transient or permanent; she still required insulin at the time of her death when she was 11 months old, but her diabetes may have been prolonged by the recurrent severe infections and cardiac failure.

Loss of maternal-specific methylation at *NESPAS*, part of the complex, imprinted *GNAS* cluster on chromosome 20q13, is also associated with a well-recognised clinical phenotype; pseudohypoparathyroidism type IB (PHP IB; MIM 603233).[14] However, features of this condition such as hypocalcaemia, hyperphosphataemia, and elevated parathyroid hormone were not seen in either sib (Table 1). We interpret the slight TSH elevation with normal T3 and T4 in the older sister on one occasion as an incidental finding. Neither sib had any features suggestive of Albright hereditary osteodystrophy (MIM 103580). The clinical consequences of hypomethylation in humans at *GRB10*, *PEG3*, and *PEG1/MEST* are not known and may account for some of the unexplained findings described in Table 1. Alternatively, these may

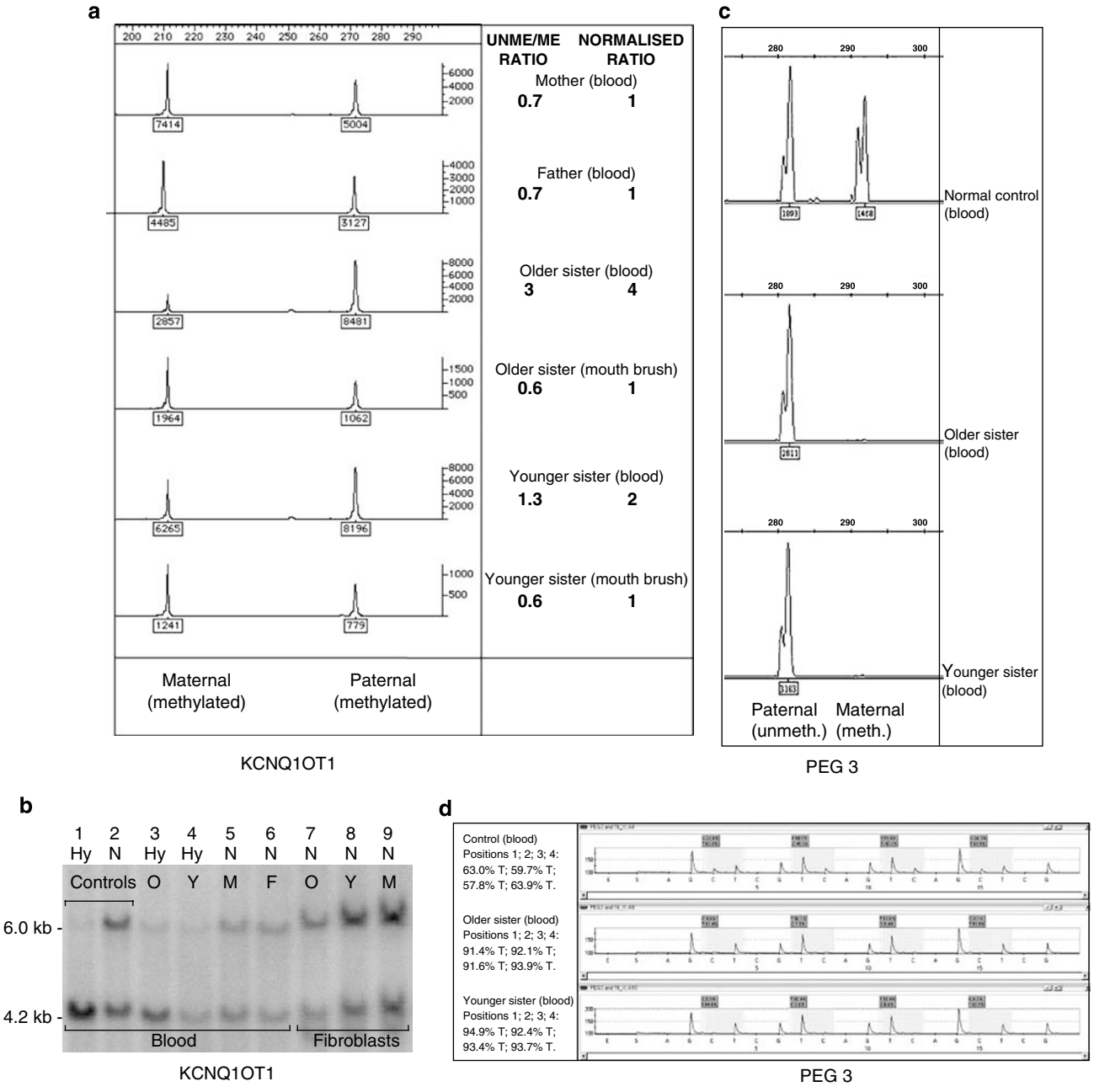

Figure 3 Examples of the methylation analyses performed. (**a**) MS-PCR analysis at *KCNQ1OT1* in BWS DMR2. X-axis: product size (bp); Y-axis: peak height. The ratio T/C was calculated as the height ratio of unmeth/meth products, and normalised to the average of normal control samples. Both sisters have hypomethylation of the maternal allele in DNA from blood and normal methylation in DNA obtained by mouth brush. (**b**) Methylation-specific Southern blot analysis of *KCNQ1OT1* (*LIT1*) in BWS DMR2. Restriction enzymes: *Bam*HI and *Not*I. Probe: LIT1 (AA155639). Hy = hypomethylation; N = normal; O = older sister; Y = younger sister; M = mother; F = father. Both sisters have hypomethylation of the maternal allele in blood-derived DNA and normal methylation in DNA from fibroblasts. (**c**) MS-PCR analysis at *PEG3*. Method similar to that used in (**a**). Both sisters have complete loss of maternal methylation. (**d**) Quantitative pyrosequencing of bisulphate-induced C/T polymorphisms (shaded areas) at *PEG3*. Both sisters have strongly elevated T/C ratios, indicating marked maternal hypomethylation.

result from methylation aberrations at loci or tissues not tested.

There are significant differences in the clinical phenotypes of the sisters; most notably, the repeated infections, epilepsy, severe psychomotor retardation, failure to thrive, and early death of the younger sister. These phenotypic differences may reflect differences in the methylation patterns in the two sibs. Alternatively, given the high

degree of consanguinity, it is possible that the severity of symptoms in the younger sister was the result of a second genetic disorder.

As regards the differential diagnosis, we considered two rare genetic conditions with methylation aberrations at multiple sites. The ICF syndrome is caused by mutations in the *DNMT3B* gene,[15] affecting the methylation and condensation of paracentromeric heterochromatin; micronuclei and centromeric instability of chromosomes 1 and 16, and, less often, 9 are typically found. ICF patients have variable types of immunodeficiency and a characteristic dysmorphic appearance with hypertelorism, protruding tongue, and micrognathia.[16] While the severe infections seen in sib 2 and macroglossia are somewhat reminiscent, the other clinical findings seen in this family are different, and no centromeric instability or abnormalities of immunoglobulins were demonstrated.

Aberrant maternal methylation at multiple imprinted loci has been demonstrated in familial recurrent biparental complete hydatidiform mole (HYDM; MIM 231090).[17,18] This is also autosomal recessively inherited. In the majority of cases, biparental complete HYDM is caused by mutations in the *NLRP7* gene (HNGC approved nomenclature; synonym *NALP7*).[19] Homozygosity in affected women causes a dysregulation of imprinting in the germ line and leads to successive moles or occasionally other reproductive losses. This represents a global maternal imprinting defect incompatible with fetal life.[20] Allelic mutations in *NLRP7* could, however, hypothetically account for the multiple maternal hypomethylation syndrome, pointing to the possibility that the gene defect in this syndrome may affect either the sibs or their mother. It must be noted that there was no history of recurrent moles or pregnancy losses in the family. Microsatellite markers and SNPs around *NLRP7* showed that the mother was heterozygous, and furthermore, sequencing her *NLRP7* exons and intron–exon boundaries revealed no mutations. That is, a *NLRP7* mutation in the mother is unlikely as the underlying defect in this family, although it is not completely ruled out. Other potential candidate genes are the DNA methyltransferases involved in the establishment or maintenance of DNA methylation: *DNMT1* and *DNMT1o* (its oocyte-specific isoform), *DNMT2*, *DNMT3A*, *DNMT3B*, and *DNMT3L*. Our analysis of SNPs and microsatellites did not point specifically to any one of these genes as a candidate for a homozygous mutation in the mother. In mice, however, embryos derived from *Dnmt1o*-deficient oocytes have been found to have loss of allele-specific expression and methylation, specifically at imprinted loci.[21] Heterozygous progeny of female mice null mutant for *Dnmt3L* have been shown to have specific demethylation of maternally methylated, imprinted genes.[22] Therefore, based on these mice data, both of these genes could be candidates, and further studies are in progress.

Clinical implications

The clinical findings in the older sister initially pointed to a diagnosis of TNDM (Table 2), which was subsequently confirmed by genetic testing. Therefore, the diagnosis of hypomethylation at other loci such as *KCNQ1OT1* was substantially delayed, although a suspicion of BWS was raised from birth. On the basis of this finding, we recommend that TNDM patients who are primarily diagnosed with LOM at 6q24, should also be tested for LOM at 11p15.5, so that if a molecular diagnosis of BWS is made, surveillance for embryonal tumours can be offered.

The risk of recurrence of this novel syndrome is not known. Initially, when only the TNDM DMR at 6q24 was known to be involved, the mother was counselled that the recurrence risk of LOM TNDM was likely to be low, as all cases so far had been sporadic, however, with the caution that LOM TNDM is a very rare condition. The birth of the second affected child and the detection of hypomethylation at further loci make it likely that the disorder in this family is genetic with a significant reproductive risk. However, the exact risk to future offspring is difficult to know. If the syndrome is caused by a homozygous autosomal recessive defect in the sisters, the risk is 25%. But if instead the mother is homozyogous for such a defect affecting the imprinting of her oocytes, the situation is comparable to biparental complete HYDM, where affected women have a theoretical recurrence risk of 100%. Selective mechanisms, however, modify this risk figure in biparental complete HYDM, as in a few families, some of the affected women have experienced normal pregnancies.[20] Finally, due to the very broad clinical spectrum in the sisters, it is currently impossible to predict the phenotype associated with multiple hypomethylation. Further knowledge about the underlying mechanisms and the associated phenotypic spectrum in multiple maternal hypomethylation syndrome is needed.

Acknowledgements

We thank the family for their collaboration. We thank the technicians at the Kennedy Center and Jonathan Callaway, Salisbury, for their laboratory work and photographer Jette B Rasmussen for excellent assistance with preparation of the figures. Deborah JG Mackay was funded by Diabetes UK.

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