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SHORT REPORT

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Monozygous triplets discordant for transient neonatal diabetes mellitus and for imprinting of the TNDM differentially methylated region

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Abstract Transient neonatal diabetes mellitus (TNDM) is associated with paternal over-expression of an imprinted locus on chromosome 6q24, which contains one differentially methylated region (DMR); maternal demethylation at the DMR accounts for ~20% of cases. Here we report female monozygous triplets, two of whom have TNDM arising from loss of maternal methylation within the TNDM DMR.

Introduction

Transient neonatal diabetes mellitus (TNDM) (OMIM 601410) affects approximately 1:400,000 infants. Growth-retarded neonates present with persistent hyperglycaemia and require insulin therapy for approximately 3 months, after which time the diabetes resolves (Temple and Shield 2002). Most TNDM cases are caused by over-expression of a paternally expressed imprinted locus on chromosome 6q24, for which three genetic causes are known: paternal UPD6 (31% of the Wessex cohort), paternal duplication of chromosome 6q24 (36%), and loss of maternal methylation of the TNDM differentially methylated region (DMR) at chromosome 6q24 (22%). In 11% of cases no molecular cause has been identified.

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Case report

Female monochorionic, triamniotic triplets were spontaneously conceived by a gravida 1, para 0 mother. Pregnancy was uncomplicated, though intrauterine growth retardation of one triplet was noted ultrasonographically. Labour was induced at 35+3 weeks gestation due to maternal hypertension. Triplets were delivered vaginally without complications, but were hospitalised because of prematurity and dysmaturity. The clinical characteristics of the neonates are recorded in Table 1. Follow-up at the age of 3 years was normal for all three triplets.

The mother of the triplets is not diabetic, nor did she become hyperglycaemic during pregnancy. Two of her paternal aunts developed diabetes in their 60s, one of whom is insulin-dependent; two sons of this aunt developed diabetes in their forties and are insulin-dependent.

Materials and methods

Extraction of genomic DNA from blood and buccal cell samples, and molecular genetic diagnosis of TNDM by microsatellite analysis, ratiometric PCR of chromosome 6q24 and methylation analysis at chromosome 6q24, were performed as described in Gardner et al. (2000). Molecular genetic diagnosis of TNDM by methylation-specific duplex PCR was performed as described (Mackay et al. 2005). Zygosity was tested using the Promega Powerplex 16 system according to the manufacturer's instructions.

Table 1 Clinical course of TNDM in the triplets

Triplet	1	2	3
Apgar score (1, 5, 10 min)	8, 9, 9	7, 9, 10	10, 10, 10
Birth weight (g)	1,900	1,564	2,090
Head circumference (cm)	32	30	32
Onset of diabetes (days)	4	2	–
Glucose (mmol/l)[a]	10.8	23.5	4.0
Insulin (mU/l)[a]	4	2	5
C-peptide (nmol/l)[a]	0.08	0.34	0.16
Remission of diabetes (days)	26	26	–

[a] Measured synchronously on day 2

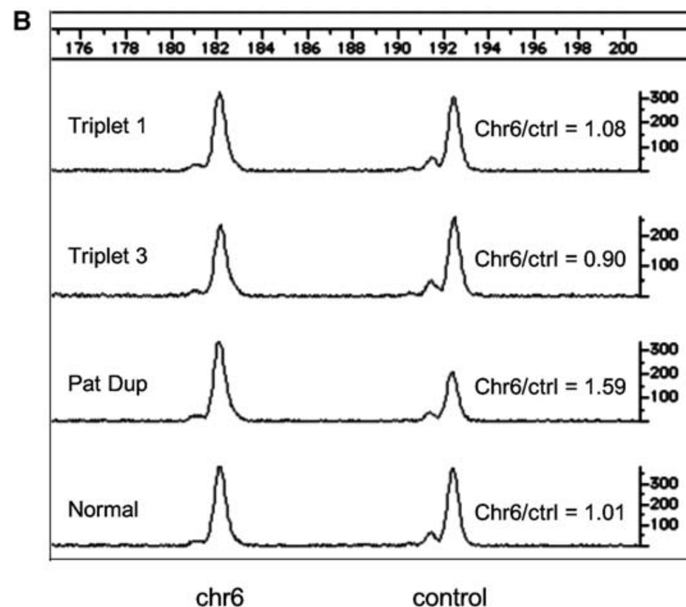
Results

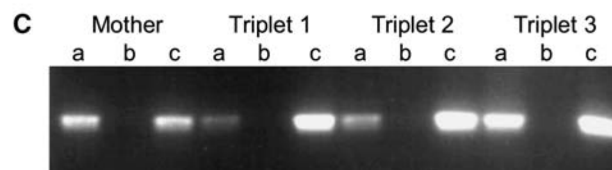
Microsatellite profiling of buccal swab DNA from the triplets showed identical biparental inheritance of six markers distributed throughout the genome, and identical inheritance of eight more which were not fully informative in the parents (data not shown); taken together with the microsatellite analysis of chromosome 6, these results indicated a very high likelihood of monozygosity. In triplets 1 and 2 with TNDM, microsatellite analysis excluded paternal UPD6, and ratiometric PCR

Fig. 1a–c Molecular diagnosis of TNDM by microsatellite analysis, ratiometric PCR and MS-PCR. Triplets 1 and 2 were affected by TNDM; triplet 3 was unaffected. **a** Results of microsatellite analysis for six informative markers on chromosome 6. Position on chromosome 6 is indicated in Mb from 6pter: the TNDM locus lies at 144 Mb from 6pter. Both blood and buccal swab DNA were analysed in order to eliminate mosaic paternal UPD6 as a cause of TNDM. The results for triplet 2 were the same as those of triplet 1. **b** Electropherograms of QF-PCR. Each duplex PCR reaction contained primers for both a sequence within the TNDM locus and a control sequence on chromosome 5, and 50 ng template DNA; after 20 cycles of PCR, the product ratio reflected the allelic ratio of the source DNA. Ratiometry was performed for three unique sequences within the TNDM locus. The results for triplet 2 were the same as those for triplet 1. **c** Blood-derived genomic DNA was digested with, **a** *Hpa*II (CCGG; methylation-sensitive, **b** *Msp*I (CCGG; methylation-insensitive), **c** mock digest. Thirty-six cycles of PCR, using primers spanning restriction sites within the TNDM DMR, were performed on digestion products, and PCR products were visualised on agarose gel. The high number of PCR cycles precludes use of this method for quantitative diagnosis; it is used for pUPD6/methylation defect identification

A

D6S	1636	1628	286	1704	264	281
Mb	55	63	80	144	166	170
triplet 1, blood	1,3	1,3	1,2	1,2	1,2	2,3
triplet 1, buccal swab	1,3	1,3	1,2	1,2	1,2	2,3
triplet 3, blood	1,3	1,3	1,2	1,2	1,2	2,3
triplet 3, buccal swab	1,3	1,3	1,2	1,2	1,2	2,3
mother	1,2	1,1	2,4	1,1	1,3	1,2
father	3,4	2,3	1,3	2,2	2,3	3,3





of chromosome 6q24 showed no evidence of paternal duplication (Fig. 1a, b). Methylation analysis, by digestion of blood-derived DNA with methylation-sensitive restriction enzymes followed by 36 cycles of PCR, demonstrated the presence of both methylated and unmethylated alleles of the TNDM DMR in all three children (Fig. 1c).

We performed ratiometric methylation-sensitive PCR (MS-PCR) of bisulfite-treated DNA to measure the ratio of methylated (C) and unmethylated (T) alleles of the TNDM DMR (Mackay et al. 2005): paternal and maternal duplications of 6q24 reproducibly give C/T ratios approximating to 0.5 and 2.0, respectively, whereas both UPD6 and 6q24 methylation-defective patients have no C allele, and therefore a C/T ratio of 0. Peripheral blood DNA from all three triplets gave anomalously low C/T ratios of approximately 0.25–0.3 (Fig. 2).

We hypothesised that triplets 1 and 2 had isolated methylation anomaly of the TNDM DMR, obscured in blood-derived DNA by foetal circulation sharing. Ratiometric MS-PCR on buccal swab DNA from triplets 1 and 2 demonstrated complete absence of a C allele, indicating an isolated methylation defect, whereas the ratio of triplet 3 was normal (Fig. 1).

Discussion

This case represents the first description of TNDM discordancy in a multiple birth, diagnosed by sensitive ratiometric MS-PCR. Isolated methylation loss at the TNDM DMR is present in 14 out of 62 kindreds in the Wessex cohort, and has so far been described only as a sporadic event.

Epigenetic discordance of twins has also been described in Beckwith–Wiedemann syndrome (BWS): Weksberg et al. (2002) demonstrated discordant BWS resulting from loss of maternal methylation in ten out of ten monozygotic twin pairs analysed, monozygotic twins constituting 8% of a BWS cohort compared with 0.3–0.4% in the general population. The Wessex TNDM cohort is too small for statistical analysis, though the presence of one set of triplets among 22 cases of methylation loss may be considered unexpected. However, this case strengthens the hypothesised causal link between imprinting errors involving maternal methylation loss and monozygotic twinning (Weksberg et al. 2002; Bestor 2003). Monochorionic monozygous twinning occurs some cell cycles later than establishment of imprinting (Machin 1996). Discordant twinning following imprinting errors may be driven by growth asymmetry between the normally and anomalously imprinted cell clones (Bestor 2003); such an asymmetry-driven mechanism could also be envisaged in TNDM, which is associated with a gross growth retardation phenotype. The discordant TNDM genotype in two out of three triplets suggests that the imprinting error may well have preceded twinning in this case.

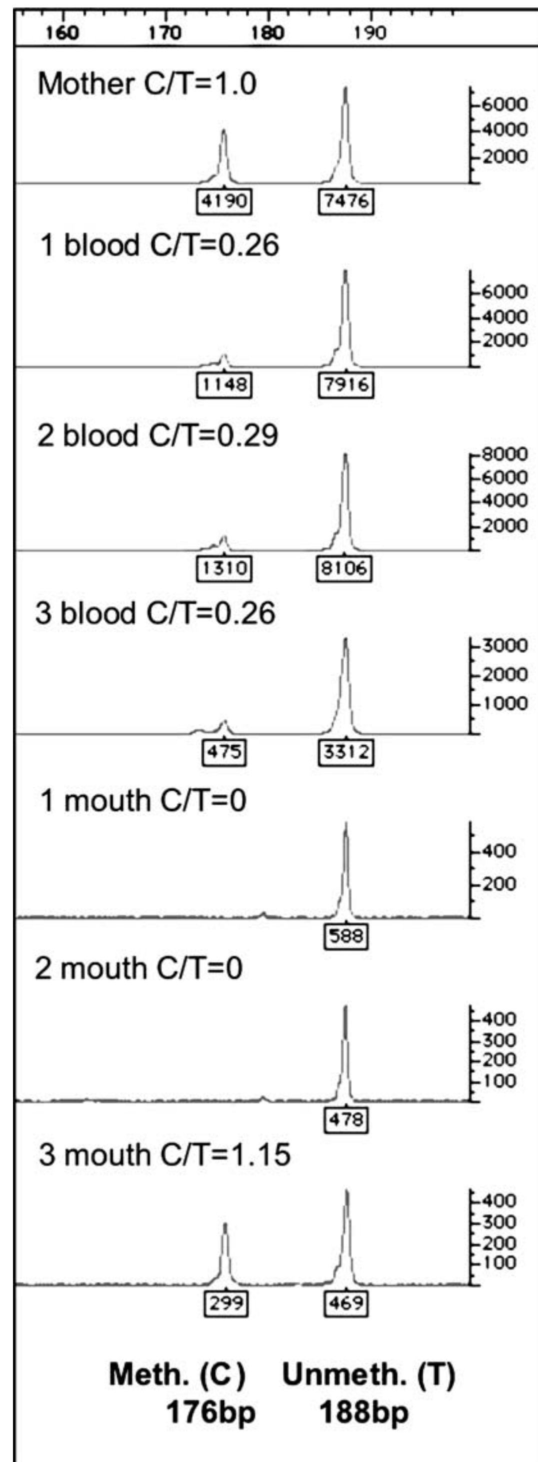


Fig. 2 Electropherograms of amplification products of MS-PCR. *x-axis* scale represents calculated product size (bp), *y-axis* indicates peak height, as do figures under each peak. The ratio (C/T) was calculated as the peak height ratio of methylated versus unmethylated amplification products and normalised to that observed in the mother of the triplets. (The methylation ratio of the mother was in turn verified as normal against five control samples; data not shown.) Blood-derived and mouthbrush-derived DNA are as indicated

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