Maternal Hypomethylation of KvDMR in a Monozygotic Male Twin Pair Discordant for Beckwith-Wiedemann Syndrome

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Beckwith-Wiedemann syndrome (BWS; OMIM 130650) was first described by Beckwith in 1963 and Wiedemann in 1964 [Beckwith, 1963; Wiedemann, 1964]. BWS is a phenotypically and genetically heterogeneous overgrowth syndrome associated with an increased risk for embryonal tumor development [Pettenati et al., 1986; Elliott et al., 1994; Weng et al., 1995] and occurs with an incidence of 1 in 13,700 live births [Weksberg et al., 2010]. Nearly 85% of reported BWS cases are sporadic, but 15% of them are familial. BWS is characterized by macroGLOSSIA and gigantism as originally described by Beckwith and Wiedemann, although other clinical characteristics as abnormal growth manifested by hemihyperplasia, and, less frequently, difficulties in feeding and speech and sleep apnea can also be found. Hypoglycemia is re-
ported in 30–50% of babies with BWS [Engstrom et al., 1988]. Children with BWS have an overall risk for tumor development estimated at 7.5% [Tan and Amor, 2006], with Wilms’ tumor and hepatoblastoma being the most common, but other embryonal tumors have also been reported, including rhabdomyosarcoma, adrenocortical carcinoma and neuroblastoma [Lapunzina, 2005]. The BWS phenotype has a wide spectrum, from hemihyperplasia and nevus flammeus to intrauterine, neonatal, or pediatric death [Weksberg et al., 2010]. Final growth parameters are usually within normal limits; however, growth parameters should be followed regularly. The differential diagnosis includes Simpson-Golabi-Behmel syndrome, Sotos syndrome, Costello syndrome, Perlman syndrome, and mucopolysaccharidosis type VI.

BWS is caused by various epigenetic and/or genetic alterations that dysregulate the imprinted genes on chromosome 11p15.5. The BWS critical region contains 2 domains: the differentially methylated region H19DMR, which regulates the expression of IGF2 and H19 in domain 1; and the differentially methylated region KvDMR, which regulates the expression of CDKN1C, KCNQ1OT1, and KCNQ1 in domain 2. In more than 80% of individuals with BWS, molecular genetic testing can detect alterations causing BWS. Loss of methylation in KvDMR on the maternal chromosome is present in around 5% of cases. Mutations of the maternal CDKN1C allele (~5% of cases with no family history, ~40% of cases with family history) have also been reported. Paternal uniparental disomy of 11p15.5 (~20% of cases) and duplication (<1% of cases) or inversion/translocation (<1% of cases) involving 11p15.5 have been described in BWS patients [Reik and Maher, 1997; Bliek et al., 2001; Cooper et al., 2005; Weksberg et al., 2005, 2010]. However, 20% of BWS patients have unknown (epi)genetic cause(s). Other genes have been demonstrated to be involved in single families with atypical BWS: the NSD1 gene was found mutated in 2 patients [Baujat et al., 2004]. Also, mutations in NLRP2 at 19q13.42 have been reported in 2 children of 1 BWS family with multilocus methylation defect [Meyer et al., 2009]. There are some genotype-phenotype correlations; for instance, the epigenetic abnormalities of H19DMR are associated with high risk for tumor development, specifically nephroblastoma [Bliek et al., 2001; Cooper et al., 2005].

An increased frequency of monozygotic (MZ) twinning has been observed in BWS patients [Weksberg et al., 2002; Bliek et al., 2009]. Moreover, twinning in BWS affects almost exclusively females, is nearly exclusively caused by hypomethylation in KvDMR and is mostly associated with discordance, i.e. one twin is affected, while the other is less or unaffected. The remarkable prevalence and discordance of BWS twins indicates that the imprinting failure is at least contemporaneous with twinning and possibly causally connected [Weksberg et al., 2002; Bliek et al., 2009].

Here we report a male MZ twin pair discordant for BWS with their detailed DNA methylation study. DNA methylation data obtained from blood and buccal swab samples of the twins and their parents are presented. We show that the phenotype is mainly due to absence of methylation at maternally imprinted KvDMR.

**Patients and Methods**

**Clinical Report**

The male twins were delivered at 36 weeks of gestation by caesarean section. At birth, the affected twin weighed 2,200 g, the unaffected twin 2,100 g. The twin pregnancy was the second pregnancy of healthy unrelated Moroccan parents. At birth of the twins, the family consisted of the 35-year-old mother, the 39-year-old father and an 8-year-old healthy brother. At 4 months, the affected twin was 62 cm in length (50th centile), his weight was 7.2 kg (75th centile), and his head circumference was 42 cm (50th centile). He had neonatal hypoglycemia. His appearance was characteristic of BWS with macrosomia, macroglossia, omphalocele, inguinal hernia, neonatal hypoglycemia, and ear creases. The psychomotor development was normal. The unaffected twin had no clinical features of BWS, with normal measurements at 4 months. His length was 58 cm (10th centile), his weight was 5.6 kg (10th centile), and his head circumference was 41 cm (25th centile). The older brother was healthy. The parents had no dysmorphic features of BWS. At a second clinical exam at age 1 year, the affected twin had a length of 76 cm (50th centile), a weight of 11 kg (75th centile) and a head circumference of 47 cm (75th centile). The unaffected twin was 74 cm in length (25th centile), his weight was 9 kg (10th centile), and his head circumference was 46 cm (50th centile). The twins were regularly monitored for weight and height; furthermore, the BWS-affected twin had abdominal ultrasound and serum alpha fetoprotein level monitoring every 3 months.

**Molecular Studies**

Informed consent was obtained from the probands’ parents prior to implementation of the genetic studies reported here. Peripheral blood and buccal epithelial cells (buccal swab) were collected from the twins at the age of 1 year (affected and unaffected) and their parents.

Genomic DNA was extracted from blood and buccal swabs using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions.

**Methylation Analysis by Methylation-Specific MLPA**

Methylation status of the 2 imprinted domains at 11p15.5 (H19DMR and KvDMR) was determined by methylation-specific MLPA (MS-MLPA) using the SALSA MLPA kit ME030-B2-BWS/
RSS (MRC-Holland, Amsterdam, The Netherlands), in accordance with the manufacturer’s instructions. MS-MLPA is a semi-quantitative method for methylation profiling. It is a variant of the MLPA technique in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme. Analysis of the MS-MLPA PCR products was performed on an ABI3500 Genetic Analyzer using the GeneMapper software (Applied Biosystems, Foster City, Calif., USA). For copy number analysis, the data generated was intranormalized by dividing the peak area of each amplification product by the total area of the reference probes only. The ratios were then obtained by dividing the intranormalized probe ratio in a sample by the average intranormalized probe ratio of all reference runs. For methylation analysis, the intranormalized peak area of each MS-MLPA probe from the digested sample was divided by the value obtained for the undigested sample.

Methylation Status Confirmation by Pyrosequencing

In order to quantify and confirm the results obtained by MS-MLPA, pyrosequencing assays were performed. First, ~500 ng DNA was subjected to sodium bisulfite treatment and purified using the EZ GOLD methylation kit (ZYMO, Irvine, Calif., USA) according to the manufacturer’s instructions. KvDMR promoter PCR amplification was performed in a 25-μl reaction containing HotStarTaq Master Mix (Qiagen), 0.1 μM forward primer KvDMR-F, 5′-TGTTTTAGTTAGTTATTTGTTG-3′, and biotinylated reverse primer KvDMR-R, 5′-CCCCATCTCTCTATAAAATAATTT-3′ (the reverse primer was 5′-biotinylated to facilitate single-strand DNA template isolation for the pyrosequencing reaction). The amplification was carried out according to the routine protocol: denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. The entire biotinylated PCR product was mixed with 40 μl of binding buffer and 2 μl (10 mg/ml) streptavidin-coated polystyrene beads. Bead-amplicon complexes were captured on a vacuum prep tool (Qiagen), and the PCR products were denatured using 0.2 M NaOH. The denatured DNA was resuspended in 0.3 μl of sequencing primer (5′-GGGTATATAGTTATTTATAGTA-3′) dissolved in annealing buffer, and primer annealing was achieved by heating the sample to 80 °C for 2 min before cooling to room temperature. The pyrosequencing reaction was carried out on a PyroMark Q24 instrument (Qiagen). The PyroMark Q24 v2.0.6.20 software automatically determines individual methylation frequencies for all CpG sites in the amplicon; the degree of methylation is calculated from the ratio of the peak heights of C and T.

Fig. 1. MS-MLPA methylation results from blood (a) and buccal swabs (b). Methylation results from pyrosequencing experiments of 8 CpG islands analyzed at KvDMR in blood and buccal swabs. White bars correspond to the median of 5 independent controls, black bars to the index patient and grey bars to the healthy twin.
Zygosity Testing

As no data on placentation was available, zygosity testing was performed on DNA isolated from blood lymphocytes using 13 polymorphic microsatellite markers (D3S3694, D7S2519, D8S277, D13S1283, D16S3276, D20S171, and D21S2055). Microsatellite typing was performed by fluorescent PCR. The results were analyzed with an ABI3500 Genetic Analyzer and Gene Mapper v.4.1 software, using Gene Scan ROX500 as an internal size standard (all from Applied Biosystems).

**Results**

The MS-MLPA study of the 11p15 region revealed hypomethylation at the maternally imprinted KvDMR in all the tissues analyzed for the affected twin, confirming the clinical diagnosis of BWS. Loss of methylation at KvDMR was also found in blood of the unaffected twin. However, when buccal swab was analyzed, a normal methylation pattern was observed. No copy number variation was identified in the tested probes for any of the samples (fig. 1a, b). These results were confirmed and quantified by pyrosequencing (fig. 1c; table 1; online suppl. fig. 1, 2; see www.karger.com/doi/10.1159/000356689 for all online suppl. material), refining that the healthy twin’s methylation status was normal.

Both parents presented a normal methylation pattern in all the analyzed tissues.

Microsatellite analysis revealed monozygotic twinning (table 2) and discarded paternal disomy (table 3).

**Discussion**

MZ twins result from fecundation of a single ovum by 1 sperm. MZ twins originate from 1 zygote and are considered to be genetically identical. The prevalence of twin births varies between populations [Zwijnenburg et al.,...
The highest prevalence of twinning is reported in Nigeria, where 1 in 12 persons is a member of a twin pair; the lowest twinning rates are found in Asia (8/1,000 births in China) [Gan et al., 2007; Hoekstra et al., 2008]. In Europe and the USA, the incidence of twinning is 1 twin pair in every 60 births [Hall, 2003; Hoekstra et al., 2008]. The rate of MZ twinning is constant at 3–4 per 1,000 maternities around the world [Tong et al., 1997; Hall, 2003]. The variation in twinning rates is generally accepted as being the result of variation in dizygotic (DZ) twinning rates. The causes of MZ twinning in humans are unknown. Shur [2009] assumed that (epi)genetic mechanisms may be involved in the phenomenon of splitting of the zygote. Recently, an increasing frequency of discordant MZ twins has been reported [Zwijnenburg et al., 2010]. Various etiologies were suggested, such as chromosomal mosaicism, point mutations, epigenetic phenomena, and many others.

A large number of BWS twins have been described since the first report in 1980 [Berry et al., 1980; Brown, 1986; Litz et al., 1988; Olney et al., 1988; Chien et al., 1990; Franceschini et al., 1993; Leonard and Johnson, 1993; Leonard et al., 1996; Machin, 1996]. Gaston et al. [2001] reported 5 multiple births: 2 pairs of MZ female twins, and 3 DZ twin pairs. The 2 MZ twin pairs both showed hypomethylation at KvDMR. A cohort of 250 BWS patients was described by Weksberg et al. [2002], the frequency of MZ twinning was 8% as compared with 1% DZ twinning. All MZ twins were phenotypically discordant, and hypomethylation of KvDMR was reported as the cause of BWS. Blike et al. [2009] reported a cohort of 13 twin pairs (12 twin pairs and 1 triplet). For 11 of the 13 cases, the genetic defect was hypomethylation of the maternal KvDMR. In 1 case, a mosaic trisomy of chromosome 11p was found to result in an increased methylation level of H19DMR and a decreased methylation level of KvDMR. In the last case, there was no detectable genetic defect. Other genetic defects have been found in BWS twins: uniparental disomy in a male MZ twin pair and a mosaic trisomy 11p15 in a female MZ twin pair [Li et al., 2001; Marcus-Soekarman et al., 2004; Smith et al., 2006].

Based on these data, we could summarize that the majority of BWS twins are MZ discordant, and most of them present hypomethylation of KvDMR. The excess of MZ twins among BWS patients with KvDMR hypomethylation suggests that the methylation defect and the twinning process are correlated [Bestor, 2003].

In most of the reports, the MZ twins are females: 12 of the 13 in the paper of Blike et al. [2009], 8 of the 10 twin pairs reported by Weksberg et al. [2002], with very few cases of male discordant twins [Weksberg et al., 2002; Smith et al., 2006; Blike et al., 2009; Tierling et al., 2011]. The striking excess of female twins suggests a possible mechanistic link with X chromosome inactivation [Orstavik et al., 1995].

Here, we report a male MZ twin pair with loss of methylation at KvDMR found in blood of the affected and unaffected twin. However, when buccal swab was analyzed, only the affected twin presented loss of methylation. Recently, Tierling et al. [2011] reported a further case of male MZ twins discordant for BWS. KvDMR hypomethylation was detected in the affected twin in all tissues, whereas for the unaffected twin, KvDMR hypomethylation occurred only in the blood and was not detected in DNA obtained from fibroblasts, saliva and buccal swabs (table 1). Values for methylation defects in swab samples were less marked, both in our patient and in the one reported by Tierling et al. Through this observation, we would like to highlight the importance of the analysis of independent tissues for clinically discordant MZ twins (or any kind of multiplex). The follow-up of our discordant BWS MZ twins will provide more clinical data (because of their short age actually) and disclose other manifestations that are not present as yet in the affected twin.

**Acknowledgements**

We thank the patients and their family. The authors thank Valeria Romanelli for technical assistance on KvDMR pyrosequencing. This work was supported by FIS-program (I3NSCA10/01056 to I.G.); I3NS Program of the Spanish Ministry of Health (CP03/0064; SIVI 1395/09 to G.P.N.), and Fundacion Eugenio Rodriguez Pascual to G.P.N.

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