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Short Communication

H19DMR methylation analysis in patients with Beckwith-Wiedemann syndrome and isolated hemihyperplasia

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Abstract

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth disorder of complex and heterogeneous etiology involving alterations in genomic imprinting. The cause of isolated hemihyperplasia (IHH) is unknown but might be due to partial or incomplete expression of BWS because both these conditions share predisposition for the same types of neoplasias. We investigated the methylation pattern of the putative imprinting control region H19DMR using peripheral blood from 12 patients, six with clinical features of BWS and six with IHH. All the patients had normal karyotypes and paternal uniparental disomy (UPD) was excluded in 10 informative cases. The normal H19DMR methylation pattern was found in eight informative patients, indicating that H19DMR methylation was not related to their condition. We suggest that the absence of neoplasias in the BWS and IHH patients studied might be related to the absence of UPD and to the presence of normal H19DMR methylation.

Key words: Beckwith-Wiedemann syndrome, isolated hemihyperplasia, genomic imprinting, DNA methylation, uniparental disomy, H19DMR.

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Beckwith-Wiedemann syndrome (BWS) is a congenital disorder characterized by overgrowth and predisposition to some types of cancer. The most common features associated with BWS are increased growth, macroglossy, ear lobe creases and/or posterior helical indentations, and abdominal wall defects (Elliott *et al.*, 1994). Children with BWS have a 7-21% risk of developing embryonic malignancies, most notably Wilms' tumor of the kidney (Weksberg *et al*., 2001). Hemihyperplasia or asymmetric growth of one or more parts of the body can be observed in approximately 12.5% of individuals with BWS and is present in 40% of BWS patients who develop tumors (Wiedemann, 1983). It has been suggested that isolated hemihyperplasia (IHH) represent partial or incomplete expression of BWS because both these conditions produce a predisposition for the same types of neoplasias (Sotelo-Avila *et al*., 1980).

The etiology of BWS is both complex and heterogeneous but is characterized by genetic and epigenetic alterations in the 11p15.5 chromosomal region which contains two genetic domains (telomeric and centromeric) regulated by genomic imprinting, a process that leads to the silencing of a specific parental allele. The *IGF2* and *H19* genes have been mapped to the telomeric domain, while the *CDKN1C* (also known as *p57Kip2*), *KVLQT1* and *LIT1* (also known as *KvLQT1AS*) genes map to the centromeric domain (Maher and Reik, 2000). It has been suggested that two imprinting control regions (ICR), H19DMR and KvDMR, control the expression of these genes. Alteration of the H19DMR methylation pattern has been associated with loss of imprinting. of the *IGF2* gene (Bell and Felsenfeld 2000), while abnormal methylation of KvDMR has been associated with loss of imprinting of the *LIT1* and *CDKN1C* genes (Diaz-Meyer *et al*., 2003).

Paternal uniparental disomy (UPD) associated with BWS results in over-expression of growth promoter genes, which are normally activated only in the paternal chromosome (Kotzot, 1999). Catchpoole *et al*. (1997) reported that UPD in the 11p15.5 region occurred in about 20% of sporadic BWS cases.

The etiology of IHH still remains unclear with most reported cases having been sporadic and without chromosomal rearrangements, although it has been suggested that IHH represents patchy over-expression of the *IGF2* gene due to defective imprinting (Hoyme *et al*., 1998). The frequency of hemihyperplasia among BWS patients with uniparental disomy or aberrant methylation of both

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H19DMR and KvDMR has been found to be significantly higher than that in patients without such defects (DeBaun *et al*., 2002).

Loss of imprinting of the *IGF2* gene leading to expression of the normally silent maternal gene seems to be an important factor in the association between neoplasias and BWS and/or IHH. The abnormal H19DMR methylation observed in some patients with BWS may be related to a predisposition to develop tumors, especially Wilms' tumor (DeBaun *et al*., 2002). Loss of imprinting of H19DMR has also been shown in patients with hepatoblastomas, rhabdomyosarcomas, and gonadoblastomas (Bliek *et al*., 2001; Weksberg *et al*., 2001).

Knowledge of the etiologic mechanisms of BWS and IHH may be useful for patient management and for better genetic counseling of their families, because of which we undertook a study to identify alterations leading to constitutional loss of imprinting in BWS and IHH patients. In this study we took blood samples from BWS and IHH patients and investigated them for chromosomal rearrangements, paternal UPD and the methylation pattern of the putative imprinting control region H19DMR.

Molecular and cytogenetic analyses were carried out on two females and four males with BWS (median age = 9 years) and three females and three males with IHH (median age = 10 years), all being outpatients at the Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The study was approved by the National Ethical Committee (CONEP) and informed consent was obtained from the participating families. Clinical diagnosis of BWS was based on three major features (macroglossia, pre or postnatal growth > 90th percentile, and abdominal wall defects) or two of these major features plus at least three minor features such as ear lobe creases or posterior helical ear pits, facial *nevus flammeus*, hypoglycemia, nephromegaly or hemihyperplasia (Elliott *et al.*, 1994). All the BWS patients showed asymmetrical growth and were apparently sporadic cases. We included in the study IHH patients who showed asymmetrical overgrowth of one limb or one side of the body (with or without facial, trunk or visceral involvement) but excluded patients with neuromuscular alterations characteristic of other disorders.

Cytogenetic analysis was performed on peripheral blood lymphocytes after GTG-banding (Scheres, 1972) and genomic DNA was obtained from the same blood samples (Olerup e Zetterquist, 1992). All patients were first screened for paternal UPD by the restriction fragment length polymorphism (RFLP) method using the *H19*/*RsaI* (Zhang *et al*., 1992) and *IGF2*/*ApaI* (Tadokoro *et al*., 1991) polymorphisms and by D11S4177 and D11S922 microsatellite analysis (http://www.ncbi.nlm.nch.gov, nucleotide access numbers Z53859 and Z16988).

The allele specific methylation pattern was evaluated by the differential methylation of *HhaI* RFLP mapped to the H19DMR region after digestion by the methylation-sensitive *Hpa*II restriction enzyme and polymerase chain reaction (PCR) amplification (Jinno *et al*.,1996). In the presence of normal H19DMR mono-allelic methylation *HpaII* digests only the unmethylated allele preventing its amplification but when bi-allelic methylation (hypermethylation) is present both alleles remain undigested and are amplified by the PCR. In non-informative cases (*HhaI* homozygotes) we also analyzed *AvaI* RFLP. Both *HhaI* or *AvaI* digestion was carried out on DNA samples previously digested with *HpaII* and on samples with no *HpaII* digestion. For the *HhaI* polymorphism the PCR was performed using the primers H1 (5' CAATGAGGTGTCCCAGTT CCA 3') and H2 (5' CACATAAGTAGGCGTGACTTGA 3') and for the *AvaI* alleles nested PCR was performed using the primers V1 (5' GAGCCTGCCAAGCAGAGCG 3') and V2 (5' CACATAAGTAGGCGTGACTTGA 3') and the internal primers N1 (5' GTGTCCCCATTCTTT GGATG 3') and N2 (5' GTTTCACACTAGGGCCGAGA 3'). Alleles were visualized using electrophoresis on 2% agarose gel and ethidium bromide staining.

None of the 12 patients studied had chromosomal alterations. The presence of paternal UPD was excluded in three patients based on analysis of the *H19/RsaI* polymorphism and the presence of a maternally inherited allele, the remaining cases being non-informative. Genotyping of the D11S4177 and D11S922 loci enabled the identification of bi-parental inheritance in 10 patients (Figure 1, Table 1). Two BWS patients were non-informative in both analyses.

Analysis of H19DMR methylation revealed a normal pattern in eight patients, 5/6 patients with IHH and 3/6 with BWS (Figure 2), the four remaining cases being non-informative due to RFLP homozygosity. A summary of the results is shown in Table 1.

The absence of chromosomal abnormalities in the BWS and IHH patients analyzed agrees with previous reports showing a low frequency (1-2%) of chromosome aberrations in BWS cases (Maher and Reik, 2000).

Paternal UPD occurs in approximately 20% of BWS cases as a result of meiotic and/or mitotic non-disjunctions (Catchpoole *et al*., 1997). Although Itoh *et al*. (2000) reported UPD mosaicism in BWS and observed that the most severely affected organs presented the highest percentage

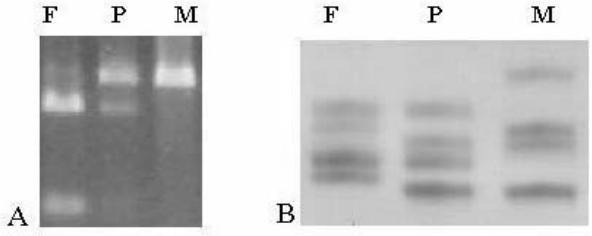

Figure 1 - Exclusion of uniparental disomy by comparison of patient and parental genotypes. Inheritance of maternal and paternal alleles as assessed by (A) *H19/RsaI* RFLP and (B) microsatellite D11S4177 loci (F = father, M = mother, P = patient).

Table 1 - Summary of the investigation of uniparental disomy (UPD) and H19DMR methylation pattern in patients with Beckwith-Wiedemann syndrome (BWS) and Isolated Hemihyperplasia (IHH).

	UPD				Methylation pattern	
Patient	*H19*/*RsaI*	*IGF2*/*ApaI*	D11S4177	D11S922	DMR/*HhaI*	DMR/*AvaI*
			BWS			
1	NI	NI	NI	NI	NI	NI
2	NI	NI	NI	NI	NI	NI
3	BP	NI	BP	BP	MAM	NA
4	NI	NI	BP	BP	MAM	NA
5	NI	NI	BP	BP	NI	NI
6	NI	NI	BP	BP	NI	MAM
			IHH			
7	NI	NI	BP	BP	MAM	NA
8	BP	NI	BP	NI	MAM	NA
9	NI	NI	BP	NI	NI	NI
10	NI	NI	BP	NI	MAM	NA
11	BP	NI	BP	BP	MAM	NA
12	NI	NI	BP	BP	MAM	NA

NI = non-informative, BP = bi-parental inheritance; MAM = mono-allelic methylation; NA = not analyzed.

of cells with UPD, Gaston *et al*. (2001) demonstrated that when UPD is present in tongue and tumor samples it was also detectable in peripheral blood and was always associated with abnormal bi-allelic methylation in the H19DMR region. In our study, the absence of UPD in 10 patients agreed with the H19DMR mono-allelic methylation seen in the eight informative patients.

It has been postulated that *IGF2* over-expression caused by loss of imprinting is one of the main factors responsible for overgrowth in BWS and IHH patients (Weksberg *et al*., 1993). We could not analyze *IGF2* expression because our patients were not heterozygous for *IGF2*/*ApaI* RFLP (Table 1), which precluded reverse transcription (RT) expression studies using RT-PCR). Because of this we opted to carry out 'indirect' expression analysis based on methylation of the putative H19DMR imprinting control region. Sporadic BWS cases with bi-allelic *IGF2* expression and bi-allelic H19DMR methylation have been reported by Reik *et al*. (1995) but in our series of patients the eight informative cases showed a normal H19DMR methylation pattern, although these patients might have alterations in the KvDMR imprinting control region as reported by Brown *et al*. (1996) in BWS patients.

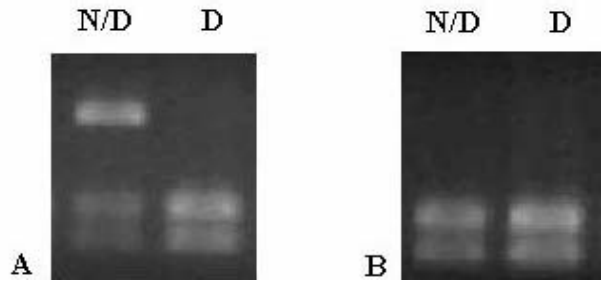

Figure 2 - The H19DMR methylation pattern as determined by *HhaI* RFLP analysis (Lane N/D = *HpaII* undigested DNA; Lane D = *HpaII* digested DNA): A) Monoallelic methylation demonstrated by the absence of the larger *HhaI* allele (upper band) in the *HpaII* digested DNA; B) Non-informative case homozygous for the smaller *HhaI* allele (see text for details).

Regarding predisposition to tumors, it has been postulated that there is a relationship between cancer risk and loss of *IGF2* imprinting and/or abnormal methylation of H19DMR because both effects have been described in neoplasias (principally Wilms' tumor) in children with BWS (Bliek *et al*., 2001). DeBaun *et al*. (2002) hypothesized that abnormal methylation in the telomeric domain (H19DMR) may be associated with overgrowth and predisposition to tumors, while abnormal methylation in the centromeric domain may be associated with macrosomy and abdominal wall defects but not with predisposition to tumors. Our patients were all at least five years old at the time of our study and have been followed up and screened for tumors at least twice a year by ultrasound, none of them having developed neoplastic processes. In spite of the small sample size, we suggest that the absence of neoplasias (especially Wilms' tumor) in the BWS and IHH patients examined in our study may be related to the absence of UPD and the presence of normal H19DMR methylation.

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