Beckwith-Wiedemann syndrome [BWS (MIM 130650)] is a genetic disease that is most frequently sporadic and characterised by somatic overgrowth, macroglossia, abdominal wall defects and a variety of secondary signs, including predisposition to embryonal tumours. Recent evidence indicates that BWS is caused by deregulation of one or more members of a cluster of imprinted genes located at chromosome 11p15.5 (Maher and Reik 2000). Imprinted genes are controlled by DNA methylation and most of them have specific DNA sequences showing differential methylation on the two parental alleles (Reik and Walter 2001). Demethylation of the maternally inherited copy of \textit{KvDMR1}, a CpG island included in an intron of the maternally expressed \textit{KCNQ1} gene, has been observed at high frequency in sporadic BWS patients (Lee et al. 1999; Smilinich et al. 1999). This epigenetic alteration has been found associated with biallelic expression of a paternally expressed gene (\textit{KCNQ1OT1}), which is transcribed in the antisense orientation with respect to \textit{KCNQ1}. This event, in turn, is believed to cause or be associated with deregulation of other linked imprinted genes, including the growth inhibitor \textit{CDKN1C}. (Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001; gene mutation nomenclature used in this article follows the recommendations of den Dunnen and Antonarakis 2001).

The extent of demethylation occurring in BWS patients at the \textit{KvDMR1} CpG island has been poorly defined so far. This was due to the use of Southern blotting coupled to digestion with methylation-sensitive restriction enzymes, as the method for methylation analysis. In this paper, we employed the bisulphite genomic sequencing procedure to determine the positions of the methyl CpGs of the \textit{KvDMR1} end proximal to the \textit{KCNQ1OT1} gene in BWS-affected and normal individuals. The results obtained demonstrated that the demethylation includes the 5′ end of the \textit{KCNQ1OT1} gene.

Methylation at \textit{KvDMR1} was first investigated by digestion with methylation-sensitive restriction enzymes and Southern blotting of the DNA extracted from the blood leucocytes of normal individuals and patients affected by BWS. The BWS patients were diagnosed on the basis of the criteria reported by Elliott et al. (1994). In addition to the previously reported \textit{NotI} site (Lee et al. 1999; Smilinich et al. 1999), two \textit{EagI} sites, located at the ends of the CpG island within two \textit{BamHI} sites, were also analysed (Fig. 1d). Fig. 1a–c shows the results obtained from representative samples of normal individuals and BWS patients. In normal individuals, a slow- (6.0 kb) and a fast-migrating (1.4, 1.7 or 3.1 kb) band with similar intensities were observed, as expected from the occurrence of differential methylation of the two alleles. In contrast, 55% (16/29) of the BWS patients analysed showed concordant hypomethylation at the \textit{NotI} and both of the \textit{EagI} sites, as indicated by the presence of only the fast-migrating bands. The DNAs of three normal individuals and three hypomethylated BWS patients were then analysed for the presence of methylcytosine in eight CpGs located 5′ and 23 CpGs located 3′ of the second \textit{EagI} site (positions 559026–559692 of the contig NT_009368), by using the bisulphite genomic sequencing method (Fig. 1d, e). The procedure used was that reported by Frommer et al.

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Fig. 1a–e Hypomethylation of the KvDMR1 CpG island in BWS. a–e Analysis of the methylation status of the KvDMR1 CpG island by digestion with methylation-sensitive restriction enzymes and Southern blotting. DNA (10 µg) extracted from peripheral blood leucocytes of BWS patients and normal individuals (control) were digested with EagI or NotI in combination with BamHI and hybridised to probe A (Smilich et al. 1999) or probe B (PCR product obtained with primers 5′-CGCTATTGGGATGGAAGTTG-3′ and 5′-AACTCAGAGCCATGATGCAG-3′). In the control, the 6.0-kb band (BamHI fragment) corresponds to the methylated maternal allele and the 1.4-, 1.7- and 3.1-kb bands (BamHI–EagI, BamHI–NotI and EagI–BamHI fragments, respectively) to the unmethylated paternal allele. d The KvDMR1 locus. The relative positions, transcription orientations and imprinting status of the CDKN1C, KCNQ1 and KCNQ1OT1 genes are reported in the upper part of the diagram. The genes expressed by the maternal allele in normal individuals are depicted above the line and those expressed by the paternal allele below the line. The KvDMR1 CpG island, the probable 5′ end of the KCNQ1OT1 gene, the relevant restriction sites and hybridisation probes are reported in the lower part of the diagram. The accession number of the genomic sequence analysed is NT_009368. e Methylation status of 31 CpGs of the KvDMR1 CpG island analysed by the bisulphite genomic sequencing procedure. The results obtained from the DNAs of three normal individuals and a representative BWS patient are shown. Open and filled circles represent unmethylated and methylated cytosines, respectively. The bent arrow indicates the extremity of the most 5′ KCNQ1OT1 EST (AA359588)