ORIGINAL ARTICLES

# Parental allele specific methylation of the human insulin-like growth factor II gene and Beckwith-Wiedemann syndrome

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**Abstract**

**In an attempt to elucidate the role of methylation in parental imprinting at the IGF-II gene locus, for which imprinting has already been described in the mouse, we undertook an allele specific methylation study of the human IGF-II gene (mapped to 11p15.5) in a control population and in patients with Beckwith-Wiedemann syndrome.**

**In control leucocyte DNA (16 unrelated adults and eight families), the maternal allele of the IGF-II gene was specifically hypomethylated, whereas no such allele specific methylation was found for either the insulin or the calcitonin genes which are located in 11p15.5 and 11p15.1, respectively. Furthermore, the IGF-II gene specific hypomethylation was localised on the 5′ portion of exon 9.**

**In the patients with Beckwith-Wiedemann syndrome in which the IGF-II gene is thought to be involved and where paternal isodisomy has been described, hypomethylation of the maternal allele was conserved in leucocyte DNA, but abnormal methylation was detected in malformed tissues where the paternal allele was also demethylated. Some specific mechanism linked to methylation therefore seems to be involved in the pathogenesis of Beckwith-Wiedemann syndrome.**

(*J Med Genet* 1993;**30**:353–62)

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Received 15 September 1992.
Revised version accepted 20 November 1992.

Various family studies have indicated that there is a link between Beckwith-Wiedemann syndrome and the 11p15 region of chromosome 11[1 2] which also carries the insulin-like growth factor II (IGF-II) gene.[3] This syndrome causes childhood malformation and is characterised by a number of congenital disorders (neonatal gigantism, omphalocele, macroglossia, visceromegaly, hemihypertrophy, and neonatal hypoglycaemia) and is frequently associated with tumours (nephroblastoma, adrenocortical carcinoma, neuroblastoma, and hepatoblastoma).[4 5] Expression of IGF mRNA has been seen to increase in a variety of tumours[6–15] and IGF-II is known to play a pivotal role in cell proliferation[16] and embryonic development.[17] In addition, DeChiara *et al*[18] have recently used homologous recombination to show that the murine IGF-II gene is subject to differential parental imprinting. The mechanisms involved in the regulation of this imprinting remain largely unknown, but some observations indicate a relationship between parental imprinting and methylation of the alleles.[18–20]

We have therefore analysed the genomes of a control population and of children with Beckwith-Wiedemann syndrome and their families in order to determine whether DNA methylation might be linked to differential parental imprinting of the human IGF-II gene.

## Patients

Twenty-seven unrelated subjects and the brothers (P3C2 and P16C2) of two patients (P3C1 and P16C1) were analysed (15 boys and 14 girls). Their clinical findings are listed in table 1 which shows the major anomalies associated with Beckwith-Wiedemann syndrome. In 16 cases (patients P1 to P15, including the sibs P3C1 and P3C2) the symptoms were those of a complete form of Beckwith-Wiedemann syndrome. For the patient P3C2, a fetal serum sample was obtained for a variety of echographic abnormalities and for karyotype analysis. Four patients (P16 to P19) had a smaller number of anomalies and were diagnosed as having an incomplete form of the syndrome. Patient P16C1 had a brother, P16C2, who had a neuroblastoma, but no symptoms of Beckwith-Wiedemann syndrome. Four other patients (P20 to P23) were not diagnosed as having the syndrome, but nevertheless presented isolated major clinical symptoms typical of it and were therefore classified as cases with isolated signs of the Beckwith-Wiedemann syndrome. Two patients exhibited macroglossia (P20 and P21), one hemihypertrophy (P22), and one an omphalocele (P23). Among the 25 patients with signs of Beckwith-Wiedemann syndrome, seven developed tumours: P1, a neuroblastoma and a nephroblastoma; P2, a nephroblastoma; P3C1, a ganglioneuroma; P3C2, bilateral nephroblastoma; P6, a nephroblastoma; P16C1, an adrenocortical carcinoma; and P16C2, a neuroblastoma. In addition to the above, four children were

*Table 1 Clinical findings in patients with Beckwith-Wiedemann syndrome and related forms.*

| | Patients with complete forms of Beckwith-Wiedemann syndrome | | | | | | | | | | | | | | | | Patients with incomplete forms of Beckwith-Wiedemann syndrome | | | | | Patients with isolated signs of Beckwith-Wiedemann syndrome | | | | Patients with isolated tumours | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 | P12 | P13 | P14 | P15 | P16 | | P17 | P18 | P19 | P20 | P21 | P22 | P23 | P24 | P25 | P26 | P27 |
| | | | C1 | C2 | | | | | | | | | | | | | C1 | C2 | | | | | | | | | | | |
| Sex | M | F | F | M | M | M | M | F | M | F | F | M | M | F | M | F | F | M | M | M | M | F | F | F | F | F | M | M | F |
| Karyotype | N | N | N | N | N | N | N | N | | | N | | | | N | | | | | N | N | | | | | | | | |
| Family history | + | + | + | + | | | + | | | + | + | | + | + | + | | + | + | | | + | + | | | | | | | |
| Macroglossia | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | | + | + | + | | | | | | | |
| Gestation (wk) | 38 | 40 | 37 | 33 | 36 | 37 | 39 | 35 | 40 | 36 | 31 | 37 | | 34·5 | 37 | 40 | 36 | | | 40 | | | 42 | | 38 | | | 40 | 38 |
| Birth weight (g) | 4300 | 5500 | 3900 | 4600 | 4500 | 3700 | 4300 | 2100 | 4000 | | 2200 | 3300 | 4300 | 2400 | 3100 | 4100 | 4100 | | 4200 | 4500 | 3700 | | 3700 | | 4300 | | | 3300 | 2800 |
| Birth length (cm) | 50 | 56 | 50 | 50 | 54 | 50 | 55·5 | 46·5 | 52 | | | 53 | | 46 | 48 | 56 | 50 | | | 54 | 50·5 | | 51·5 | | | 50 | | 50 | 46 |
| Gigantism (postnatal) (SD) | | +3 | | | | +2 | +3 | | +2 | +2 | +2 | +3 | | | +1·5 | +3 | | | +3 | +3 | | | +2 | +2·5 | | | | | |
| Neonatal hypoglycaemia | + | | | + | + | + | | + | | + | | | + | + | + | + | | | + | | | | | | | | | | |
| Visceromegaly | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Liver | + | + | | + | + | | + | | + | | | + | + | + | | | | | + | + | + | | | | | | | | |
| Kidney | + | + | + | + | + | | + | | | | | + | | + | | | | | | | + | | | | | | | | |
| Spleen | + | + | + | + | | | + | | | | | | | | | | | | | | | | | | | | | | |
| Abdominal wall defect | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Omphalocele | | | + | | | + | | | | | | | + | | | | | | | | | | | | + | | | | |
| Umbilical hernia | + | | | | | | + | | | | + | | | | + | + | | | | + | | | | | | | | | |
| Diastasis recti | + | + | | | | | + | + | + | | + | | | | | | | | | + | | | | | | | | | |
| Hemihypertrophy | + | | | | | | | | | + | | | | | | | | | | | | | | + | | | | | |
| Facial abnormality | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ear | + | + | + | | | + | + | + | | | | | + | + | + | | | | | | + | | | | | | | | |
| Naevus flammeus | | | | | | | | + | + | + | + | | | + | + | | | | | | + | | | | | | | | |
| Mid-face hypoplasia | | | | | | + | | | + | + | + | | | | | | | | | | | + | | | | | | | |
| Tumours | + | + | + | + | | | + | | | | | | | | | | + | + | | | | | | | | + | + | + | + |
| Genital abnormality | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cryptorchidism | | | | | | + | | | | | | + | + | | + | | | | | + | + | | | | | | | | |
| Clitoromegaly | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Other abnormality | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Kidney-urinary | + | | + | + | | + | | | | | | + | | | | | | | | | + | | | | | | | | |
| Skeletal | + | + | + | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| Hydramnios | + | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |

+ = present. N = normal.

investigated with no signs of Beckwith-Wiedemann syndrome, but with embryonic tumours often associated with the syndrome, such as adrenocortical carcinoma (P24), neuroblastoma (patients P25 and P26), and nephroblastoma (P27).

Family analysis was possible in 14 cases, both parents of P1, P3, P5, P6, P7, P9, P10, P13, P14, P16, P19, P20, and only the mothers of patients P2 and P24.

## Controls

The control population has been described previously[15]: 16 unrelated subjects and eight families (including 10 children and 16 parents) were studied.

## Materials and methods

### SAMPLES

Blood samples from controls and patients were collected in anticoagulant (EDTA) and stored at −80°C until use for DNA extraction. Tissues, either from tumours (P1, P24) or from partial glossectomy (P1, P6 to P10, P13, P14, P20), were removed from surgical patients, frozen immediately in liquid nitrogen, and stored at −80°C until use.

### ISOLATION OF DNA AND RNA FROM BLOOD AND TISSUES

Leucocyte DNA was isolated as previously described.[21] Total DNA and RNA were extracted simultaneously from frozen tissue (for the nine samples mentioned above except for P13 for which only DNA analysis was available) according to Chirgwin *et al*[22] with some modifications as previously described.[15] Spectrometry at 260 and 280 nm was used for DNA and RNA quantification. Nucleic acid integrity was routinely checked by ethidium bromide staining.

### PROBES

The human placental IGF-II cDNA insert P35 (vector λgt 10)[23] was subcloned in plasmid PGEM4 (pTG 3907). The 663 bp *Eco*RI fragment containing 15 bp of the 5′ untranslated region (exon 6), the coding region, and 99 bp of the 3′ untranslated region (exons 7, 8, 9) of the IGF-II cDNA was used as probe. Numbering of exons was based on the nomenclature for the IGF genes as published.[24] An exon 7 specific probe (a 159 bp fragment) was obtained by *Eco*RI/*Ava*II digestion, and an exon 9 probe (a 356 bp fragment) by *Sal*I/*Eco*RI digestion of the 663 bp cDNA insert.

The human insulin 2·7 kb *Bgl*II-*Xho*I fragment was kindly provided by P Holthuizen[25] and the 827 bp human calcitonin cDNA probe by A Julienne.[26]

DNA probes were labelled with $\alpha^{32}$P-dATP by random priming (kit, Amersham, England) to a specific activity of $2 \times 10^9$ cpm/μg DNA, as indicated by the manufacturer.

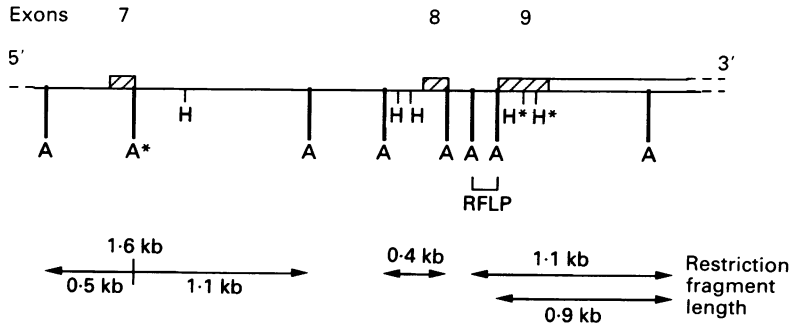


*Figure 1 Map of the relationship between AvaII and HpaII sites on the IGF-II gene around exons 7, 8, and 9; hatched areas correspond to the cDNA probe used. A = AvaII site: CCA(orT)GG. A* = specific AvaII site, methylation sensitive, depending on tissue of origin.[15] H = HpaII site: CCGG. H* = specific HpaII site, sensitive to parental allele specific methylation. The fragments detected by Southern blotting after AvaII digestion are shown at the bottom.*

### SOUTHERN BLOT ANALYSIS

Ten micrograms of DNA were digested with 120 units of restriction endonuclease, according to the manufacturer's specifications (Appligene, France or New England Biolabs Inc, USA). DNA fragments were separated by electrophoresis on 0·7% to 1·2% agarose gels, then denatured and transferred to a nylon membrane (Gene Screen Plus, New England Nuclear, USA) using the alkaline method. Finally they were hybridised to $^{32}$P-labelled probes as previously described.[21]

Enzymes were selected for Southern blot analysis on the basis of the polymorphic patterns yielded: *Ava*II and *Sac*I for the IGF-II gene,[15] *Sac*I and *Taq*I for the insulin gene,[27] and *Taq*I for the calcitonin gene.[28]

Double digests were used to study allele methylation (fig 1). The initial enzyme was used to detect polymorphism and to distinguish the alleles from each other. The second digestion was done with a methylation sensitive enzyme, *Hpa*II, through which methylation of the CCGG site could be determined by comparison with the fragments obtained with its methylation insensitive isoschizomer *Msp*I.

A specific profile was detected with *Ava*II for the IGF-II gene in some tissues where DNA was demethylated and the 1·6 kb fragment was cleaved to a 0·5 kb fragment, as previously described[15] (fig 1).

### DOT BLOT AND NORTHERN BLOT ANALYSIS

For dot blot analysis, RNA samples were dotted onto a Hybond-N membrane (Amersham, England) using a Hybri-dot (BRL, USA) apparatus as previously described.[15] For Northern blot analysis, denatured total RNA was loaded onto 1·2% agarose-formaldehyde gels for electrophoresis, then transferred to Hybond-N membranes (Amersham, England), as previously reported.[15]

The blots were hybridised according to the manufacturer's instructions with $3 \times 10^6$ cpm/ml of $^{32}$P labelled probe, then washed twice at room temperature and once at 65°C, each for 20 minutes, in 0·1 × SSPE (1 × SSPE = 0·01 mol/l $NaH_2PO_4$, pH 6·8, 0·001 mol/l EDTA-$Na_2$, 0·15 mol/l NaCl) and 0·1% SDS, before being exposed to *x* ray film (Curix RP1-

Agfa) at −80°C with intensifying screens (Dupont Cronex Hiplus).

## Results

### ALLELE SPECIFIC METHYLATION OF THE IGF-II GENE IN CONTROL LEUCOCYTES

In order to determine whether DNA methylation is linked to differential imprinting of the IGF-II gene, Southern blot analyses were done, using a system of double digestion to identify the methylation state of each of the alleles of the IGF-II gene, as described above. Sixteen unrelated adults were investigated, all of whom were heterozygous with *Ava*II for the IGF-II gene. As can be seen from a comparison of the *Ava*II digest and the *Ava*II/*Msp*I double digest shown in fig 2, all *Ava*II fragments (1·6, 1·1/0·9, 0·4 kb) were cleaved by *Msp*I, indicating that all contained some CCGG sequence. The *Ava*II/*Hpa*II double digest, by contrast, showed a conserved *Ava*II profile, but with major differences in the polymorphic 1·1/0·9 kb fragments. Both alleles were capable of cleavage by *Hpa*II, but in all 16 samples examined, only one allele was cleaved, whether it was the 1·1 kb (nine samples, 56%) or the 0·9 kb allele (seven samples, 44%). This indicates specific hypomethylation of only one allele.

The same analyses were done in eight informative families (10 children and 16 parents), and in all cases it was the maternal allele that was hypomethylated. Two examples are shown in fig 3.

In order to localise the *Ava*II fragments detected by Southern blotting, hybridisation experiments were done with IGF-II exon 7 and exon 9 specific probes. As shown in fig 4, the 1·6 kb *Ava*II fragment was specifically detected by the exon 7 probe, whereas the polymorphic 1·1/0·9 kb fragments were detected by the exon 9 probe represented in fig 1. The maternal allele specific hypomethylation site was therefore localised at exon 9 of the IGF-II gene.

### ALLELE METHYLATION OF THE INSULIN AND CALCITONIN GENES IN CONTROL LEUCOCYTES

The same methodology was used to investigate methylation of two other genes localised on the short arm of chromosome 11, insulin (11p15.5) and calcitonin (11p15.1). For the insulin gene, both alleles were partially demethylated, indicating an absence of allele specific demethyla-

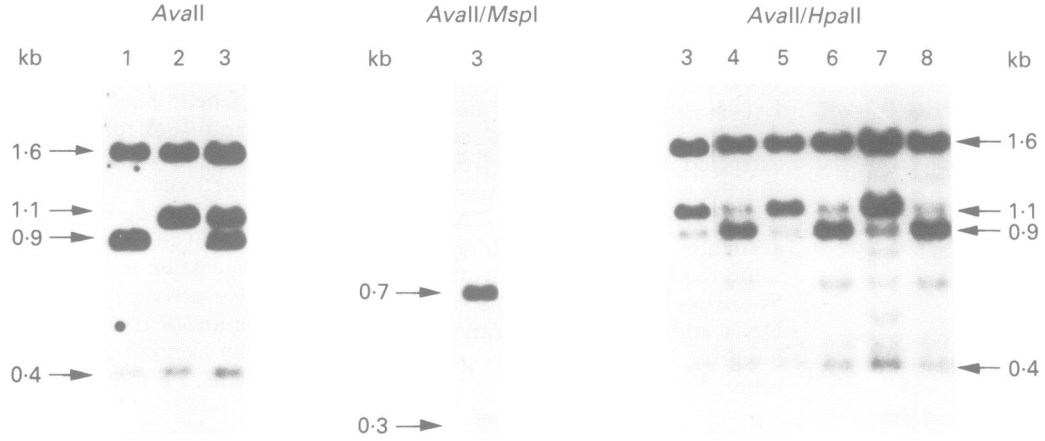


*Figure 2 Southern blot analysis of control leucocyte DNA digested with AvaII, AvaII/MspI, or AvaII/HpaII, using IGF-II cDNA as probe. (1) Homozygote for the 0·9 kb fragment. (2) Homozygote for the 1·1 kb fragment. (3, 4, 5, 6, 7, 8) Unrelated heterozygotes for the 0·9/1·1 kb fragments.*

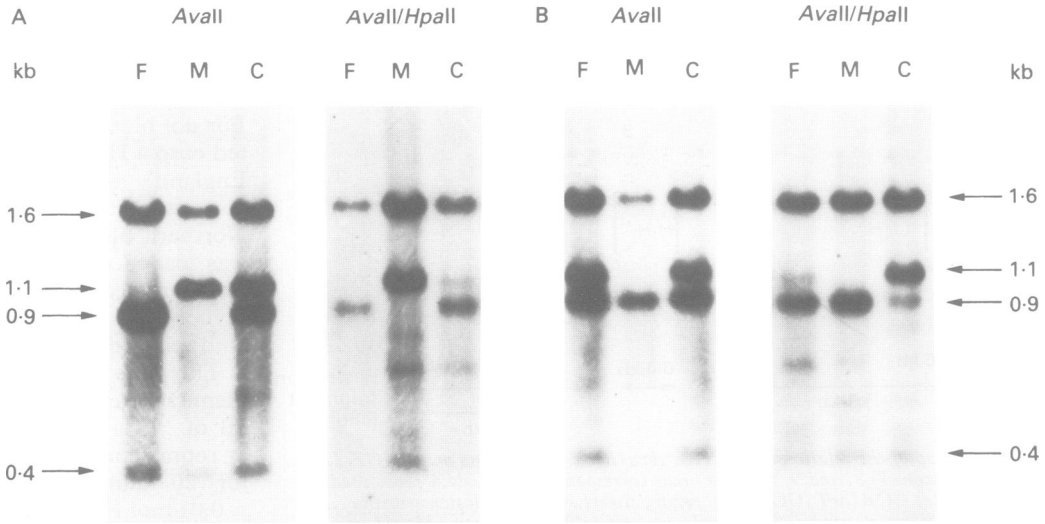


*Figure 3 Family study: Southern blot analysis of leucocyte DNA digested with AvaII and AvaII/HpaII, using IGF-II cDNA as probe. Two control families (A and B) were examined. F, father; M, mother; C, child.*

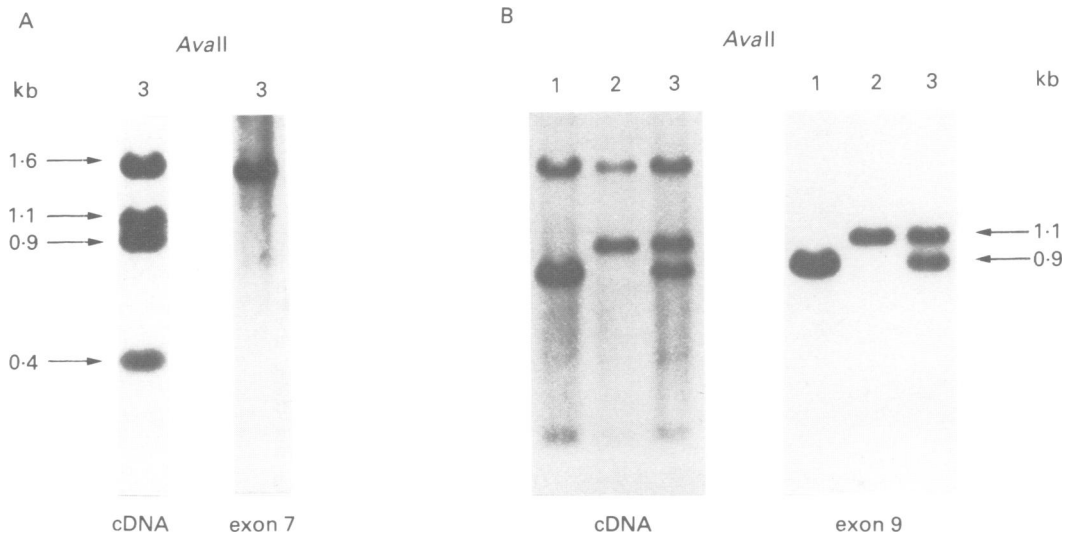


*Figure 4 Southern blot analysis of control leucocyte DNA digested with AvaII, using the IGF-II cDNA probe (exons 7, 8, 9) and exon 7 (A) or exon 9 (B) specific probes. (1) Homozygote for the 0·9 kb fragment, (2) homozygote for the 1·1 kb fragment, (3) heterozygote for the 0·9/1·1 kb fragments.*

tion, at least in the region of the polymorphic fragments (data not shown). In the case of the calcitonin gene, double digestion with *Taq*I/*Hpa*II yielded two polymorphic fragments which were shorter than those obtained after *Taq*I alone, indicating total demethylation of both alleles, without any allele specificity for the polymorphic fragments studied (fig 5).

## ANALYSIS OF THE INSULIN/IGF-II LOCUS IN PATIENTS WITH BECKWITH-WIEDEMANN SYNDROME

The genome of children with Beckwith-Wiedemann syndrome was analysed in order to determine whether abnormalities were implicated at the insulin/IGF-II locus. Leucocyte DNA was analysed in the 29 patients listed in table 1, 20 of whom were diagnosed as having Beckwith-Wiedemann syndrome (P1–P19, including P3C1 and P3C2). In addition, samples of tissue DNA (following partial glossectomy or tumorectomy) were examined when they were available. *Ava*II, *Sac*I, and *Taq*I were used to analyse the restriction fragment length polymorphisms (RFLP) at the insulin/IGF-II locus as mentioned above. For the calcitonin gene, a control gene distal to the insulin/IGF-II locus, *Taq*I was used. Where possible, the study was extended to family analyses.

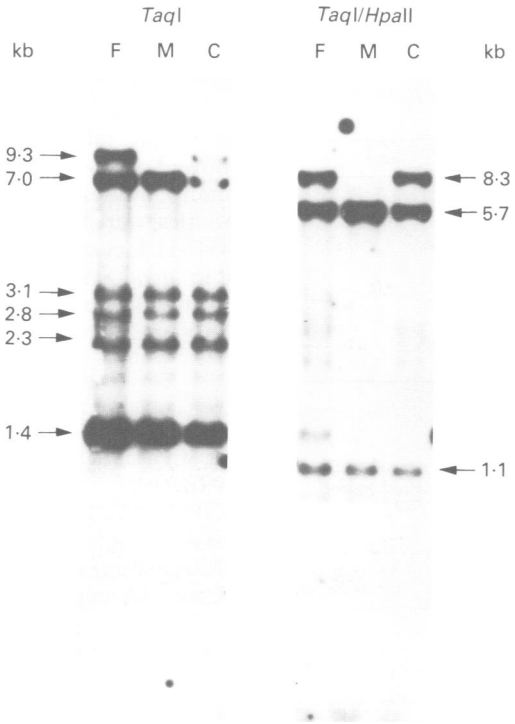


*Figure 5 Southern blot analysis of control leucocyte DNA from a control family, using the calcitonin cDNA probe with TaqI or TaqI/HpaII. F, father; M, mother; C, child.*

Among the 29 patients examined, seven were homozygous for the insulin/IGF-II locus and therefore non-informative (P4, P6, P10, P20, P22, P26, and P27) (data not shown). Out of the 22 informative cases, five exhibited profiles with abnormalities (P1, P3C1, P3C2, P9, and P24, as described in detail below and in table 2) and the remaining 17 had normal heterozygote profiles for either the IGF-II gene (P2, P11, P13, P15, P16C1, P16C2, P18, P19, P21, P23) or the insulin gene (P5, P7, P8, P12, P14, P16C1, P17, P18, P19, P23). Allele frequencies of *Ava*II and *Sac*I RFLPs at the IGF-II gene were the same as those of the control population, both in the group of 15 unrelated children with Beckwith-Wiedemann syndrome and in the group of 19 cases considered to have complete or incomplete Beckwith-Wiedemann syndrome (table 3). Furthermore, if the four contiguous loci, *Ava*II, *Sac*I for the IGF-II gene, and *Taq*I, *Sac*I for the insulin gene, were taken together, the numbers of homozygotes and heterozygotes were no different from the controls (table 4).

In four out of the 16 cases with Beckwith-Wiedemann syndrome (P1, P3C1, P3C2, and P9) abnormal heterozygote profiles were detected for the insulin/IGF-II locus. These are represented in table 2 and shown in fig 6 where disproportionate allele intensities were detected, suggesting uniparental disomy for

*Table 2 Genotypes of some patients with either Beckwith-Wiedemann syndrome or isolated tumours. Southern blot analysis of the IGF-II gene (AvaII and SacI), insulin gene (SacI and TaqI), and calcitonin gene (TaqI).*

| Restriction endonuclease (alleles (kb)) | | | IGF-II *Ava*II A: 1·1 B: 0·9 | IGF-II *Sac*I A: 10·0 B: 7·6 | Insulin *Sac*I A: 7·5 B: 6·0 | Insulin *Taq*I A: 5·6 B: 4·5 | Calcitonin *Taq*I A: 8·0 B: 6·5 | |
|---|---|---|---|---|---|---|---|---|
| | Patient | | | | | | | Associated tumour |
| Patients with complete forms of Beckwith-Wiedemann syndrome | P1 | F | A/A | B/B | A/B | A/B | A/A | |
| | | M | A/B | A/B | B/B | B/B | B/B | |
| | | C | A/b | a/B | A/b | A/b | A/B | |
| | | To | A/b | a/B | A/b | A/b | A/B | |
| | | Tu | A/A | B/B | A/A | A/A | A/B | Nephroblastoma |
| | | Nk | A/b | b/B | A/b | A/b | A/B | |
| | P3 | F | A/A | B/B | B/B | B/B | A/B | |
| | | M | A/B | A/B | A/B | A/B | B/B | |
| | | C1 | A/b | a/B | a/B | a/B | B/B | Ganglioneuroma* |
| | | C2f | A/b | a/B | a/B | a/B | B/B | Bilateral |
| | | C2 | A/b | a/B | a/B | a/B | B/B | nephroblastoma* |
| | P9 | F | A/B | A/B | B/B | B/B | A/A | |
| | | M | A/B | | A/B | A/B | B/B | |
| | | C | A/B | A/B | A/B | A/B | A/B | |
| | | To | a/B | A/b | a/B | a/B | A/B | |
| Patient with isolated tumour | P24 | M | B/B | | B/B | B/B | B/B | |
| | | C | A/B | A/B | B/B | B/B | B/B | Adrenocortical |
| | | Tu | A/b | a/B | B/B | B/B | B/B | carcinoma |

F = father. M = mother. C = affected child. To = tongue tissue after partial glossectomy. Tu = tumour after tumorectomy. Nk = normal kidney.
C1 and C2 were two affected children from the same family (see table 1), C2f being a fetal sample from the child C2.
Alleles are represented as A and B.
A/b or a/B: disproportionate profile, the less abundant allele being printed in lower case.
* Tissue not available.

the insulin/IGF-II locus. In the nephroblastoma of P1, the maternal allele (0·9 kb) was totally missing (fig 6). Allele loss was also detectable in leucocyte DNA in P1, but a heterozygote profile with disproportionate intensities was visible in tongue (fig 6) and healthy kidney tissue (data not shown). In P3C1 and her brother, P3C2 (in fetal P3C2f and newborn P3C2 leucocyte DNA), heterozygote profiles were detectable, but allele intensities were also disproportionate (fig 6). P9 had a normal heterozygote leucocyte DNA profile, but a disproportionate profile for tongue tissue (fig 6). In addition, a 0·5 kb fragment was detected in tissue samples (tongue and tumour) after *Ava*II digestion, as previously detected in a number of other tissue samples.[15] This was attributable to a specific *Ava*II site being methylation sensitive, depending on the tissue concerned (fig 1).

Among the four children with isolated tumours (P24–P27, table 1), tumoral DNA was available in only one case, P24, and an abnormal profile with disproportionate intensity was detected for the insulin/IGF-II locus (table 2). For this particular patient, the similar abnormal profile was detected in the adrenocortical carcinoma, but not in leucocyte, DNA (fig 6).

For these five patients (P1, P3C1, P3C2, P9, and P24, table 2), family analyses always showed a maternal origin of the partially or totally missing allele. The disproportionate profiles were located only at the insulin and IGF-II loci and did not extend to the calcitonin gene which, in the informative cases, exhibited a normal profile (patients P1 and P9, table 2).

*Table 3 Allele frequencies of AvaII and SacI polymorphisms at the IGF-II locus (11p15.5) in Beckwith-Wiedemann syndrome patients.*

| Alleles | *Ava*II A: 1·1 kb | *Ava*II B: 0·9 kb | *Sac*I A: 10·0 kb | *Sac*I B: 7·6 kb |
|---|---|---|---|---|
| Complete forms of Beckwith-Wiedemann (n = 15) | 70·0% | 30·0% | 36·7% | 63·3% |
| Complete and incomplete forms of Beckwith-Wiedemann (n = 19) | 68·4% | 31·6% | 36·8% | 63·2% |
| Controls* (n = 37) | 75·0% | 25·0% | 35·0% | 65·0% |

* Control population previously reported.[15]

*Table 4 Homozygote and heterozygote frequencies of four RFLPs associated with Beckwith-Wiedemann syndrome, insulin (TaqI,SacI) and IGF-II (SacI,AvaII) loci taken together.*

| | Frequency of homozygosity | Frequency of heterozygosity |
|---|---|---|
| Complete forms of Beckwith-Wiedemann (n = 15) | 20% | 80% |
| Complete and incomplete forms of Beckwith-Wiedemann (n = 19) | 16% | 84% |
| Controls* (n = 34) | 18% | 82% |

* Control population previously reported for the four contiguous loci (insulin/*Rsa*I, insulin/*Hin*dIII, IGF-II/*Sac*I, IGF-II/*Ava*II).[36]

### IGF-II mRNA EXPRESSION IN TISSUE MATERIAL FROM PATIENTS WITH BECKWITH-WIEDEMANN SYNDROME

IGF-II mRNA expression was analysed in the tissues available to determine whether any disregulation was involved. On average, IGF-II mRNA expression, measured by dot blot analysis in the tongue tissue of the eight patients having undergone partial glossectomy (P1, P6, P7, P8, P9, P10, P14, P20), was similar to that in normal adult liver used as a reference (fig 7A and B). In addition, there was no significant difference between the two cases of macroglossia where uniparental disomy had been detected (P1 and P9) and the others analysed. Moreover, the usual 6, 4·8, and

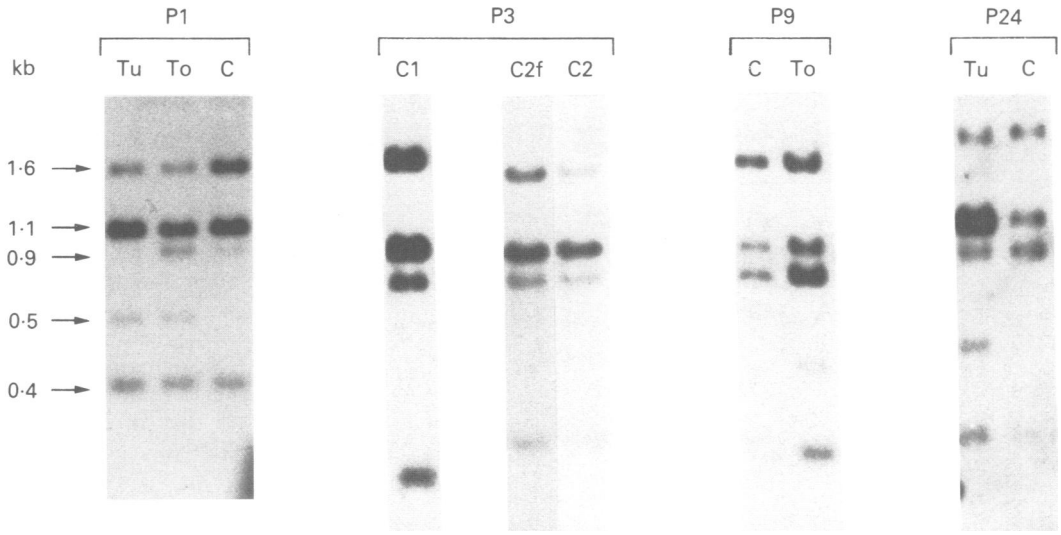


*Figure 6 Southern blot analysis of genomic DNA digested with AvaII using IGF-II cDNA as probe for patients P1 (Tu: tumour, To: tongue, C: leucocytes), P3 (C1: leucocytes, C2f: fetal leucocytes, C2: newborn leucocytes), P9 (C: leucocytes, To: tongue), and P24 (Tu: tumour, C: leucocytes).*

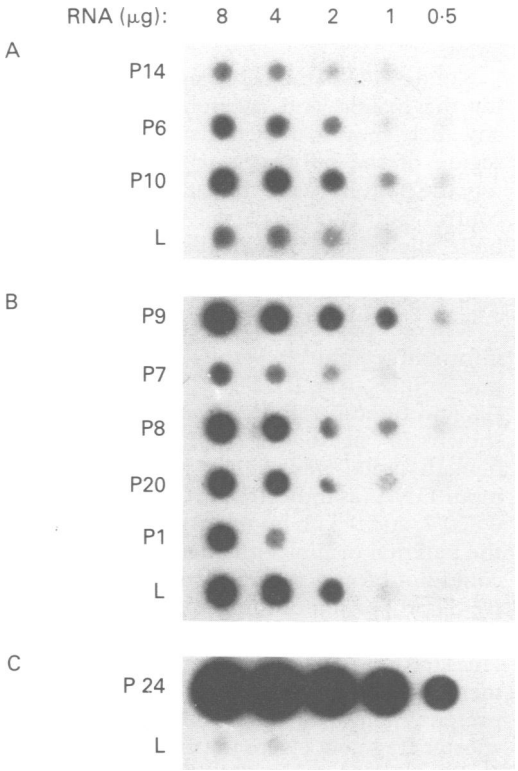


*Figure 7 Dot blot analysis of IGF-II mRNA expression in tongue tissue (P1, P6–P10, P14, P20) from patients with macroglossia and in an adrenocortical carcinoma (P24). In each experiment normal adult liver (L) was used as internal control. Exposure time: A = six days, two intensifying screens; B = four days, one intensifying screen; C = 16 hours, two intensifying screens.*

2·2 kb IGF-II mRNAs were detectable by Northern blot analysis (data not shown).

In the two samples of tumour tissue available, IGF-II mRNA expression could not be measured in the nephroblastoma of P1, who was undergoing chemotherapy at the time of surgery (data not shown), but in the adrenocortical carcinoma of P24, who was not under chemotherapy, extremely high levels of IGF-II mRNA expression were found (fig 7C).

### ALLELE SPECIFIC METHYLATION IN PATIENTS WITH BECKWITH-WIEDEMANN SYNDROME

In order to determine whether allele specific methylation of leucocyte DNA may be linked to the Beckwith-Wiedemann syndrome, double digestion experiments were done and comparisons made with the control population. This type of analysis was possible only for subjects with heterozygous profiles for *Ava*II for the IGF-II gene. In 11 of these patients (P1, P3C1, P9, P11, P13, P15, P16C1, P18, P19, P21, P23), the patterns were similar to those for the control population, indicating that only one allele was hypomethylated (data not shown). In only one case (P2) did both alleles remain methylated (fig 8). In the five informative families of Beckwith-Wiedemann syndrome patients (P1, P2, P3, P13, and P19) specific hypomethylation was always found for the maternal allele (data not shown), as had

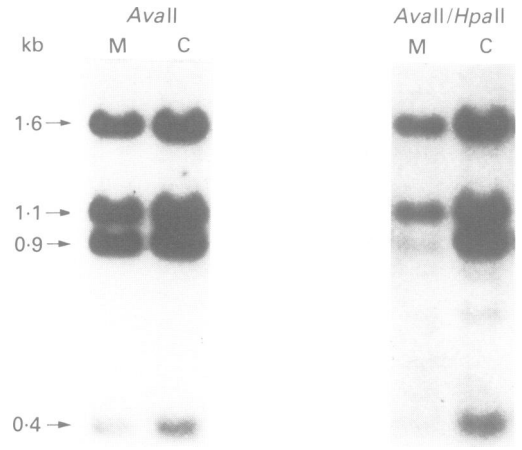


*Figure 8 Southern blot analysis of patient P2 leucocyte DNA digested with AvaII or AvaII/HpaII, using IGF-II cDNA as probe. M, mother; C, affected child.*

previously been observed for the control population.

None of the patients or families investigated for the insulin and calcitonin genes showed any allele specific demethylation at least in the region of the polymorphic fragments. Here, too, the patterns were the same as those for the control population: partial demethylation of both alleles for the insulin gene and total demethylation of both alleles for the calcitonin gene (data not shown).

Where possible, tissue samples from informative subjects were also analysed (tongue samples from P1, P9, P13 with macroglossia, and tumour samples from P1 and P24), as shown in fig 6. For P1, IGF-II DNA demethylation was evident in tongue and tumour DNA (as indicated by the presence of the 0·8, 0·7, and 0·3 kb fragments, fig 9). However, the parental origin of the demethylated alleles could not be confirmed because of the extensive disproportion of the alleles (fig 6). For this patient, P1, it was possible to show with *Ava*II/*Hpa*II digestion that the 0·9 kb fragment (which was maternal, table 2) was still present in tongue tissue, indicating that the shorter fragments were in part paternal (fig 9). In P9 and P13, both alleles (1·1 and 0·9 kb) in tongue tissue were demethylated, whereas leucocyte DNA still exhibited allele specific hypomethylation, only the 1·1 kb allele in P9 or the 0·9 kb allele in P13 being demethylated (fig 9). For P24, without Beckwith-Wiedemann syndrome, both alleles of the adrenocortical carcinoma DNA were hypomethylated (fig 9). These preliminary findings for DNA methylation in the tissues of four informative subjects showed a difference between leucocyte and tissue DNA in patients with Beckwith-Wiedemann syndrome and the patient with an embryonic tumour, whereas allele specific demethylation in control tissues (placenta, peritumoral adult kidney tissue) was similar to that in control leucocyte DNA (data not shown).

## Discussion

Allele specificity of human IGF-II gene methylation as related to parental origin has been investigated in both a control population and patients with Beckwith-Wiedemann syndrome where the IGF-II gene is thought to play a role.[1 2 29] Differential parental imprinting of the IGF-II gene has been shown in the fetal mouse,[18] but the molecular mechanism remains an enigma, despite transgenic studies suggesting that DNA methylation may be involved.[30]

Our analyses of control leucocyte DNA methylation in the IGF-II gene have shown that allele specificity does indeed exist, since only one allele was demethylated in all cases. Family analyses showed that the hypomethylated allele was always maternal, which means that allele specific hypomethylation at the IGF-II gene is linked to parental origin. Moreover, allele specificity of methylation in the 11p15 region of the chromosome was limited to the IGF-II locus (11p15.5). The phenomenon was not observed either at the insulin gene[31] (this study) or at the calcitonin gene (this study), both alleles being demethylated simultaneously, either partially (insulin) or totally (calcitonin) in the area studied. Finally, we found the sites involved in this allele specific methylation of the IGF-II gene to be situated in the region of exon 9.

In their homologous recombination studies of transgenic mice which were heterozygous

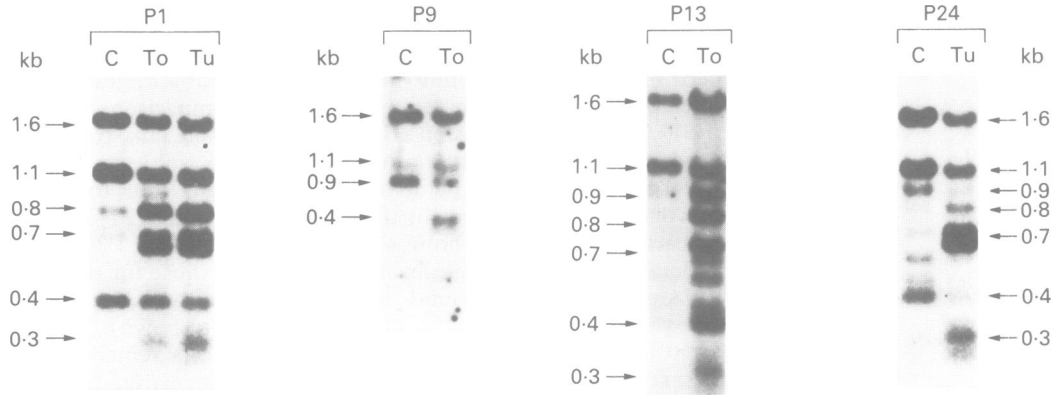


*Figure 9 Southern blot analysis of genomic DNA digested with AvaII/HpaII, using IGF-II cDNA as probe, for patients P1, P9, P13, and P24. C = leucocytes from affected child, To = tongue, Tu = tumour (P1: neuroblastoma, P24: adrenocortical carcinoma). The AvaII patterns for the same patients are shown in fig 6.*

for the mutant gene, DeChiara *et al*[18] showed that only the paternal allele of the IGF-II gene was expressed embryonically, whereas the maternal allele remained silent. Our observations indicate that in man, allele specific methylation of the IGF-II gene varies with differential parental imprinting, the maternal allele being hypomethylated and the paternal allele methylated. The allele specific IGF-II gene methylation may be linked to mRNA expression, demethylation of a gene frequently being associated with expression.[32] Nevertheless, our results suggest that the paternal allele is methylated at IGF-II exon 9 and is associated with expression of the paternal allele. This, as DeChiara *et al*[18] proposed, may reflect negative imprinting, expression of a specific *trans* acting repressor inhibiting expression of the maternal, and thereby allowing expression of the paternal, allele.

We also examined the possibility of genetic abnormalities accounting for the Beckwith-Wiedemann syndrome. Southern blotting experiments using enzymes which show RFLPs showed that in these patients (table 1) no major chromosomal anomalies (like deletion or amplification) occurred in the 11p15 region.

Loss of heterozygosity has been described in tumours where the short arm of chromosome 11 (11p13–11p15) is involved,[33] and particularly in Wilms' tumour.[34 35] Henry *et al*[36] reported increased leucocyte homozygosity in patients with the Beckwith-Wiedemann syndrome as compared to controls, but our findings do not support this, as we found the same frequency of homozygosity in the two groups[37] (tables 3 and 4). This discrepancy may be explained by the fact that some apparent allele losses in fact reflect mosaics with grossly disproportionate alleles, which simulate homozygosity in some patients (as in patient 1, fig 6). Among the 'complete' Beckwith-Wiedemann syndrome patients examined in this study, three (P1, P3C1, P9) out of 15 unrelated subjects (20%) (and four out of 16 when including P3C2) exhibited total allele loss or marked disproportion, and in all of them the maternal allele was lost (or very diminished) making duplication of the paternal allele possible. Uniparental disomy is probably only one of the mechanisms that accounts for Beckwith-Wiedemann syndrome. Among the cases of paternal isodisomy (P1, P3C1, P9), two out of three unrelated children developed tumours (66%). A malignant tumour rate of 7·5% has been reported for Beckwith-Wiedemann syndrome.[5] The high rate found in cases of uniparental disomy (with a disproportionate pattern) could be important in genetic counselling as regards the risk of tumour in Beckwith-Wiedemann patients. Nevertheless, uniparental disomy has also been seen in non-tumoral tissue (enlarged tongue and leucocytes), which means that allele loss is not always accompanied by tumour formation in these particular tissues[37] (this study). For two patients (P1 and P9), we were able to show that the mosaic disomy was limited to the distal 11p region (calcitonin not included). The presence of disproportionate allele intensities was probably the result of mosaicism for two types of cell, the normal (with normal heterozygote DNA) and the paternal isodisomy cells. This therefore concerns a post-zygotic event, with mitotic recombination, and indicates a low recurrence risk for a second child in a Beckwith-Wiedemann syndrome family. It also throws some light on the genetic mechanism involved. The recurrence of somatic mosaicism in the two sibs (P3C1 and P3C2) was highly improbable and suggests that an abnormal paternal 11p15 chromosome was transmitted.

As in the control population, allele specificity of IGF-II gene methylation was observed in leucocyte DNA of patients with Beckwith-Wiedemann syndrome. Hypomethylation of the maternal allele and methylation of the paternal allele were seen in both groups. In only one case, P2, were both constitutive leucocyte alleles abnormally methylated, whereas insulin and calcitonin gene methylation resembled that of normal controls. Here, both IGF-II alleles could have been submitted to similar paternal imprinting, suggesting a mechanism different from loss of the maternal allele with paternal duplication.

Tissue (tongue or tumour) DNA methylation in patients with Beckwith-Wiedemann syndrome proved to be different from leucocyte DNA methylation. In these patients the paternal allele of the IGF-II gene was demethylated, whether or not loss of heterozygosity had occurred. The abnormal paternal allele methylation may therefore be specific to children with Beckwith-Wiedemann syndrome and embryonic tumours.

Our study indicates that there is a link at the level of the adult human IGF-II gene between methylation and differential parental imprinting. It may be that methylation is involved in uniparental disomy and hence in the pathogenesis of Beckwith-Wiedemann syndrome. Finally, the exon 9 region of the IGF-II gene, where parental allele specific methylation is found, may play a role in regulating IGF-II gene expression.

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Contrat de Recherche Clinique de l'Assistance Publique (CRCAP 89-57, CRCAP 923107), the Fondation pour la Recherche Médicale-92, and the Université Paris VI. H Schneid is a recipient of a fellowship of the Fondation pour la Recherche Médicale. We are grateful to Dr M Binoux for his support and to Dr M Mannens for helpful discussion. We thank Drs J J Baudon, A Bensman, P Brun, I Grapin, M Gruner, G Lasfargues, M Maes, A Masson, and R Rappaport for donating samples for analysis.

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