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Relaxation of Insulin-like Growth Factor 2 Imprinting and Discordant Methylation at KvDMR1 in Two First Cousins Affected by Beckwith-Wiedemann and Klippel-Trenaunay-Weber Syndromes

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Beckwith-Wiedeman syndrome (BWS) and Klippel-Trenaunay-Weber syndrome (KTWS) are different human disorders characterized, among other features, by tissue overgrowth. Deregulation of one or more imprinted genes located at chromosome 11p15.5, of which insulin-like growth factor 2 (*IGF2*) is the most likely candidate, is believed to cause BWS, whereas the etiology of KTWS is completely obscure. We report a case of BWS and a case of KTWS in a single family. The probands, sons of two sisters, showed relaxation of the maternal *IGF2* imprinting, although they inherited different 11p15.5 alleles from their mothers and did not show any chromosome rearrangement. The patient with BWS also displayed hypomethylation at *KvDMR1*, a maternally methylated CpG island within an intron of the *KvLQT1* gene. The unaffected brother of the BWS proband shared the same maternal and paternal 11p15.5 haplotype with his brother, but the *KvDMR1* locus was normally methylated. Methylation of the *H19* gene was normal in both the BWS and KTWS probands. Linkage between the insulin-like growth factor 2 receptor (*IGF2R*) gene and the tissue overgrowth was also excluded. These results raise the possibility that a defective modifier or regulatory gene unlinked to 11p15.5 caused a spectrum of epigenetic alterations in the germ line or early development of both cousins, ranging from the relaxation of *IGF2* imprinting in the KTWS proband to disruption of both the imprinted expression of *IGF2* and the imprinted methylation of *KvDMR1* in the BWS proband. Analysis of these data also indicates that loss of *IGF2* imprinting is not necessarily linked to alteration of methylation at the *KvDMR1* or *H19* loci and supports the notion that *IGF2* overexpression is involved in the etiology of the tissue hypertrophy observed in different overgrowth disorders, including KTWS.

Introduction

The molecular mechanisms underlying human syndromes associated with overgrowth, apart from specific hormone disorders, are largely unknown (Weaver 1994). Most of the available data derive from studies on Beckwith-Wiedemann syndrome (BWS [MIM 130650]), a disease characterized by generalized or regional overgrowth, macroglossia, and abdominal wall defects, as well as a predisposition to embryonal tumors. One or more imprinted genes located at 11p15.5 are likely to be responsible for BWS (Li et al. 1997; Reik and Maher 1997). Genomic imprinting is a mechanism that causes the expression of a gene to be dependent on the gametic origin (Tilghman 1999). Evidences of its involvement in the determination of BWS include uniparental paternal disomy of chromosome 11p15, duplication of the paternally derived 11p15.5, relaxation of imprinting of the insulin-like growth factor 2 (*IGF2*) gene, and translocations of maternal chromosome 11 with breakpoint in 11p15.4-15.5 in sporadic cases of BWS. In addition, linkage to 11p15.5 with increased penetrance associated with maternal transmission is observed in the rarer familial cases (Li et al. 1997; Reik and Maher 1997). Since *IGF2* is normally transcribed only from the paternally inherited allele, overexpression of this gene might represent the endpoint of the majority of BWS molecular alterations. IGF-II, the peptide encoded by the *IGF2* gene, is a broad-spectrum mitogen with an important role in the control of prenatal growth, as demonstrated in mice with gene disruption and growth retardation, or in animals expressing high levels of this growth factor

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and showing somatic overgrowth at birth (De Chiara et al. 1990; Leighton et al. 1995; Eggenschwiler et al. 1997; Sun et al. 1997). For these reasons, *IGF2* overexpression could be responsible for the tissue hypertrophy observed in BWS. Consistent with this hypothesis, Wilms tumor, rhabdomyosarcoma, and hepatoblastoma, which are frequently associated with BWS, show high levels of *IGF2* mRNA as well as similar genetic and epigenetic defects related to the 11p15 region (Toretsky and Helman 1996). However, alteration of other linked imprinted genes may contribute to some of the symptoms of BWS (Reik and Maher 1997).

Altered DNA methylation at two loci of chromosome 11p15.5 has frequently been observed in patients with BWS (Joyce et al. 1997; Lee et al. 1999; Smilinich et al. 1999); 5%–10% of patients with BWS show hypermethylation and silencing of the otherwise active maternal allele of the *H19* gene. These individuals also display biallelic *IGF2* expression, consistent with the hypothesis that the linked *IGF2* and *H19* genes compete for the same enhancer elements (Reik and Maher 1997). More frequent (30%–50%) is the demethylation of the maternally inherited copy of *KvDMR1,* a CpG island included in an intron of the paternally imprinted *KvLQT1* gene, a locus located 350 Kb centromeric to *IGF2* (Lee et al. 1999; Smilinich et al. 1999). This epigenetic alteration, the significance of which is still undefined in the BWS pathogenesis, has been found to be associated with biallelic expression of a maternally imprinted gene (*KvLQT1-AS* or *LIT1*) transcribed in the antisense orientation with respect to *KvLQT1* (Lee et al. 1999). A subset of BWS cases with *KvDMR1* hypomethylation have loss of *IGF2* imprinting, but each of the two epigenetic lesions has also been found individually (Lee et al. 1999; Smilinich et al. 1999).

IGF-II turnover is controlled by binding to the membrane-associated type II–IGF receptor (Rechler and Nissley 1990). In a minority of human individuals, the *IGF2R* gene is subjected to genomic imprinting, since only the maternal allele is expressed (Xu et al. 1993). Mice deficient in *Igf2r* activity have elevated levels of circulating IGF-II and are 25%–30% larger than their normal sibs, raising the possibility that individuals who have monoallelic expression of this gene may have increased susceptibility to overgrowth disorders and malignancy (Lau et al. 1994).

Klippel-Trenaunay-Weber syndrome (KTWS [MIM 149000]) is a disease of unknown etiology characterized by cutaneous hemangiomata and regional hypertrophy of bones and soft tissues. In this article, we describe a family in which two first cousins are affected, one by BWS and one by KTWS, and we provide evidence of partially overlapping epigenetic alterations in the two patients. The results indicate a link between the molecular defects leading to BWS and to KTWS and provide new information on the relationship among various 11p15.5 loci.

Subjects and Methods

Patients

The BWS proband was diagnosed on the basis of the presence of hypoglycemia, exomphalos, and macroglossia. Clinical evaluation of the boy at 6 years of age revealed weight and height at the 90th–95th percentile, mild right hemihypertrophy, and hepatosplenomegaly. Analysis of ultrascan showed enlargement of the pancreas and kidneys at the upper limits of the normal range. Neither ear lobe grooves nor circular depressions of posterior helices were observed. The other described patient with BWS (BWS2) was macrosomic at birth and had visceromegaly and macroglossia. Clinical examination of the boy at 1 year of age also revealed left hemihypertrophy and bilateral Wilms tumor.

At 32 weeks of gestation, the KTWS proband presented with hydrothorax, which required intrauterine thoracentesis. At birth, diffuse capillary hemangiomata and hemihypertrophy of the left side were observed. Clinical evaluation of the boy at age 7 years indicated that weight and height were in the 75th percentile. We also observed marked hemihypertrophy of both bone and soft tissues of the left side, including the left side of the tongue and the maxillary bone, and diffuse hemangiomata on both sides. No visceromegaly was found by ultrasound examination. The presence of arterovenous fistulae was ruled out.

Cell Cultures

Cultured skin fibroblasts were obtained by means of a punch biopsy and established by standard techniques. Cells used for the analysis of allele-specific expression were of low passage number (<10th generation).

Isolation of DNA and RNA

Genomic DNA from blood leukocytes and cultured skin fibroblasts was prepared by SDS–proteinase K digestion and phenol-chloroform extraction methods. Total RNA was purified from cells by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987) and further treated for 30 min with 1 U of RQ RNase-free DNase (Promega) at 37°C in the presence of 40 mM Tris-HCl, pH 7.6, 10 mM NaCl, 6 mM $MgCl_2$, and 100 μM CaCl2.

Allelic Analysis

The *Apa*I restriction fragment–length polymorphism (RFLP) of the *IGF2* gene was analyzed by PCR and

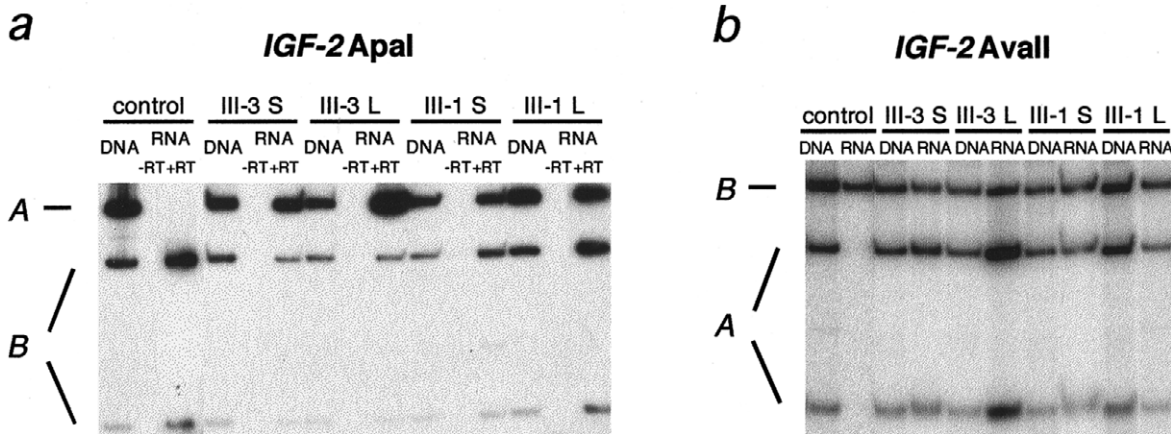

Figure 1 Relaxation of *IGF2* imprinting in the related BWS and KTWS patients. *IGF2* alleles were analyzed by PCR and RT-PCR in DNA and RNA extracted from cultured skin fibroblasts derived from the BWS (III-I) and KTWS (III-3) patients and a control individual by means of primers specific for the *Apa*I RFLP. Fibroblasts derived from the larger (L) and smaller (S) sides of the bodies were analyzed. The *Apa*I RFLP was typed with the *Apa*I (*a*) and *Ava*II (*b*) restriction enzymes. For the *Apa*I RFLP, the allele uncut by *Apa*I and cut by *Ava*II is indicated with the letter A, and the allele cut by *Apa*I and uncut by *Ava*II is indicated with the letter B. RNA was tested with (+RT) and without (−RT) addition of RT to check for contamination by genomic DNA.

reverse transcriptase (RT)–PCR as described elsewhere (Pedone et al. 1994), except for the addition of $\alpha[^{32}P]$-dATP to the reaction mixture. The *Apa*I RFLP was also typed with the *Ava*II restriction enzyme. The 5′ and 3′ parts of the variable number of tandem-repeated DNA sequences (VNTR) present in the *IGF2* gene were analyzed after labeling of the sense or antisense primer, respectively, and after digestion of the amplification product with *Bst*UI (Rainier et al. 1993). The VNTR of the tyrosine hydroxylase gene (*TH*) and the *H19 Rsa*I and *Alu*I RFLPs were typed as described elsewhere, with the addition of $\alpha[^{32}P]$-dATP to the reaction mixture (Edwards et al. 1991; Casola et al. 1997). The VNTR of the 3′ UTR of the *IGF-2R* gene was typed as described by Hol et al. (1992) by end labeling one of the primers. All RNA samples were run in duplicate with or without addition of RT to control for contamination by genomic DNA. Alleles were quantified by computer analysis of the image by a Molecular Dynamics phosphorimager.

Methylation Analysis

High–molecular-weight DNA extracted from blood leukocytes was digested with methylation-sensitive restriction enzymes, blotted, and hybridized to $\alpha[^{32}P]$-labeled probes specific for the *H19* 5′ flanking and *KvLQT1* intron 10 differentially methylated region (DMRP), as described elsewhere (Morison et al. 1996; Smilinich et al. 1999). The *H19* probe was a PCR fragment generated from human genomic DNA with the primers 5′-ACTCTGTCCTGCGGAAACCG-3′ and 5′-GGAGACAGGGCTGAGCATTG-3′ and cloned into pGEM4Z (Promega). The probes were $[^{32}P]$-labeled by random priming (Feinberg and Vogelstein 1984). Bands were quantified by analysis of the image by a Molecular Dynamics phosphorimager.

Chromosome Analysis

High-resolution chromosome analysis (600–650 bands) was performed according to the procedure reported by Gosden et al. (1982).

Results

The probands were two boys born to two sisters; one was diagnosed with BWS and the other with KTWS at birth. We analyzed the imprinting status of the *IGF2* gene in their cultured skin fibroblasts. To distinguish the paternal from the maternal allele, we checked to see whether the findings were informative for the *Apa*I RFLP (Tadokoro et al. 1991) present in the 3′ UTR of the *IGF2* gene. Since findings for both patients were informative for this polymorphism, allele-specific expression was analyzed by RT-PCR from RNA extracted from the cultured skin fibroblasts (fig. 1). The *Apa*I RFLP can also be typed with *Ava*II; the sequences recognized by the two restriction enzymes are mutually exclusive in the *IGF2* alleles (Brown et al. 1996). PCR products were therefore digested with both *Apa*I and *Ava*II, to avoid misinterpretation of the results because of incomplete digestion (fig. 1*a* and 1*b*). Results showed that both parental *IGF2* alleles were expressed in cells derived from the BWS (III-1) and the KTWS (III-3) patients (see fig. 2 for the pedigree). Since the patients showed hemihypertrophy, skin biopsy specimens were obtained from

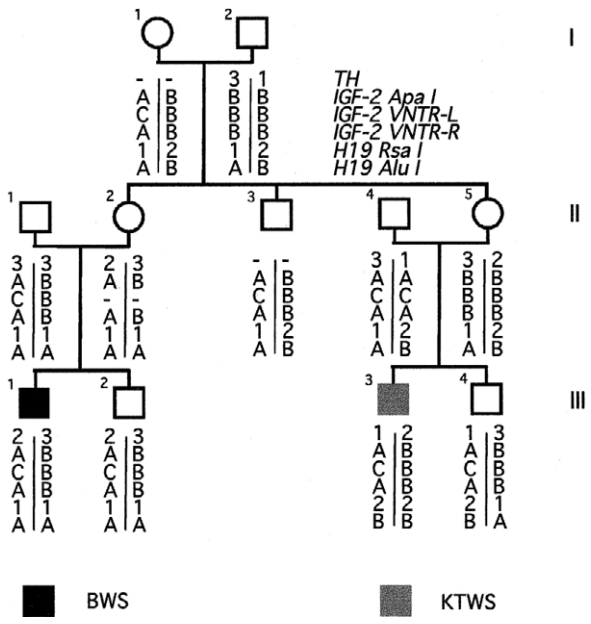

Figure 2 Pedigree and genotypes of the analyzed family for six 11p15.5 markers.

different body regions. *IGF2* expression from both parental alleles was observed in cells derived from both the larger and smaller sides of the bodies of the two patients (fig. 1*a* and 1*b*). In contrast, cells derived from seven additional informative individuals who were either healthy or affected by unrelated pathologies and whose cells were cultured under identical conditions showed monoallelic expression of the *IGF2* gene (fig. 1*a* and 1*b*; lanes from control individuals and data not shown).

To assess whether the relaxation of *IGF2* imprinting status might be due to the inheritance of a genetic defect linked to chromosome 11p15.5, we analyzed six polymorphic markers located in this region in the DNAs extracted from peripheral blood leukocytes of the patients and their relatives (fig. 2). The resulting haplotypes indicated that the BWS and KTWS patients inherited different alleles from their mothers at all loci examined from the *TH/H19* region of 11p15.5, thus excluding a common genetic defect linked to this chromosomal region. In addition, the same haplotype was inherited by both the patient with BWS and his unaffected brother. High-resolution chromosome analysis of the patient's fibroblasts was also performed and did not reveal any rearrangement of the 11p and other chromosomal regions (data not shown).

The methylation status of the *H19* and *KvDMR1* loci was then investigated in the probands (fig. 3). For this purpose, high–molecular-weight DNA was digested with *Rsa*I and the methylation-sensitive enzyme *Hpa*II and was then hybridized to a probe specific for the *H19* 5′ flanking or digested with *Eco*RI and the methylation-sensitive enzyme *Not*I and hybridized to a probe (DMRP) specific for the *KvLQT1* intron 10, as described elsewhere (Morison et al. 1996; Smilinich et al. 1999). As shown in figure 3*a*, the *H19* promoter was digested by *Hpa*II at the same extent (~50%) in the samples derived from the probands (III-1 and III-3), their unaffected brothers (III-2 and III-4), and an unrelated normal individual (C), indicating that the methylation of the *H19* alleles was unaltered in these patients. In contrast, complete methylation of the *H19* promoter was observed in an unrelated patient with BWS (BWS2). Different results were obtained when the *KvDMR1* methylation was assayed in the two cousins (fig. 3*b*). Only the BWS proband (III-1) showed a complete demethylation of the *Not*I site, whereas the patient with KTWS (III-3) displayed a 47% methylation, indicating that the demethylation of the maternal *KvDMR1* allele occurred only in the BWS proband. A pattern of partial methylation (42%–48%) was observed with the DNAs derived from the unaffected brothers (III-2 and III-4), the parents of the BWS proband (II-1 and II-2), and an unrelated normal individual (C), although the BWS proband shared with his brother the maternal and paternal haplotypes at 11p15.5 (fig. 2). Patient BWS2, who showed *H19* hypermethylation, had a normal methylation pattern at the *KvDMR1* locus, consistent with results for other BWS cases reported elsewhere (Smilinich et al. 1999).

We then investigated the inheritance of the *IGF2R* alleles and their imprinting status in the probands and their relatives by typing a VNTR present in the 3′ UTR of this gene (Hol et al. 1992). *IGF2R* expression was found to be biallelic in the cells derived from the patient with KTWS, as well as two informative unrelated patients with BWS and two control individuals (fig. 4; data not shown). Analysis of the BWS patient of the family under study did not provide information about this polymorphism. The analysis of the other family members showed that the probands and the unaffected brother of the patient with KTWS inherited the same *IGF2R* allele from their mothers, thus excluding the possibility that a defective maternal *IGF2R* allele could have been inherited by the two cousins (fig. 4).

Discussion

This work describes two boys, first cousins, one affected by BWS and the other by KTWS. Both showed relaxation of the imprinted expression of the *IGF2* gene but differed in the methylation status of the *KvDMR1* locus. The latter remained normal in the boy with KTWS but was altered in the boy with BWS. The probands inherited different alleles at the 11p15.5 loci we investigated, indicating that a common genetic defect linked to this

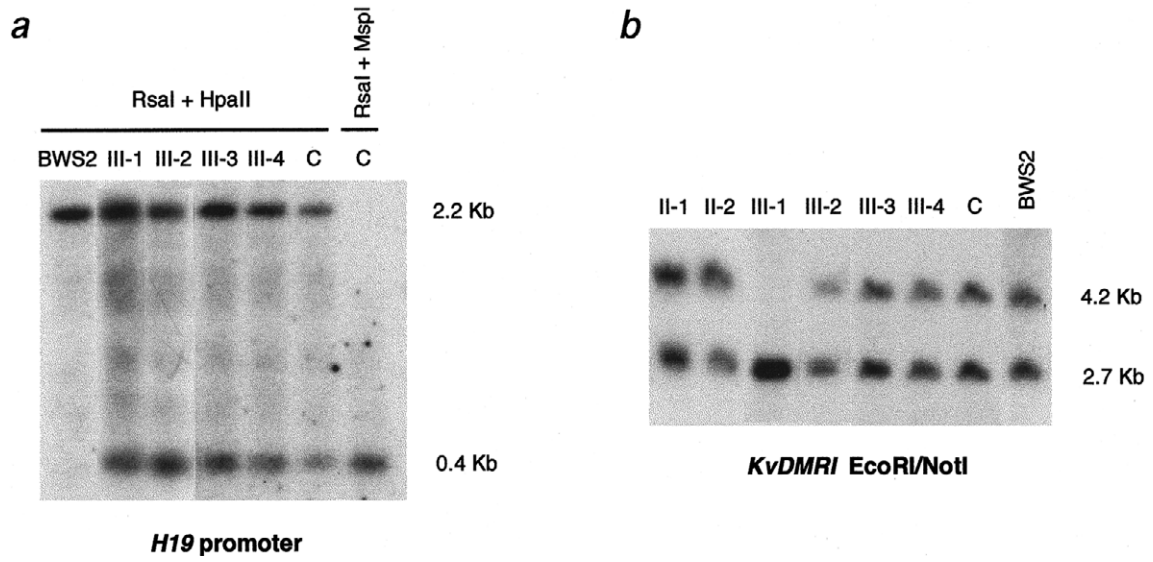

Figure 3 Demethylation of the *KvDMR1* locus in the patient with BWS. The methylation status of the *H19* promoter (*a*) and the *KvDMR1* locus (*b*) was analyzed in the DNA extracted from peripheral blood leukocytes of the BWS (III-1) and KTWS (III-3) probands, their unaffected brothers (III-2 and III-4), patient BWS2, and an unrelated normal individual (C). *KvDMR1* methylation was also tested in the parents of the BWS proband (II-1 and II-2). For this purpose, 10 μg of DNA was digested with *Rsa*I and *Hpa*II or *Rsa*I and *Msp*I and hybridized to the *H19* probe or digested with *Eco*RI and *Not*I and hybridized to the DMRP probe. The 2.2- and 0.4-Kb bands correspond to the methylated paternal and unmethylated maternal *H19* alleles, and the 4.2- and 2.7-Kb bands correspond to the methylated maternal and unmethylated paternal *KvDMR1* alleles, respectively, in normal individuals.

chromosome region was unlikely. Allelic and imprinting analysis also excluded involvement of the *IGF-2R* gene.

Relaxation or loss of *IGF2* imprinting is a relatively frequent finding in human cancer and BWS (Reik and Maher 1997; Feinberg 1999). Expression of the *IGF2* gene from both parental alleles has been observed in skin biopsy specimens derived from both sides of the body in BWS patients with hemihypertrophy, suggesting that the epigenetic defect is constitutional in these individuals (Weksberg et al. 1993). This is consistent with our findings. Apart from the observation that a BWS patient carrying a maternally transmitted 11p15.5 translocation shows biallelic expression of the *IGF2* gene, no other mutation has so far been associated with loss of *IGF2* imprinting (Brown et al. 1996). In mice, *Igf2* imprinting is disrupted by deletion of a region differentially methylated on the two parental alleles and located 5′ of the linked *H19* gene (Thorvaldsen et al. 1998). No mutations of such regions have been identified in BWS or cancer (Reik and Maher 1997). However, hypermethylation of the 5′ flanking region of the maternal *H19* allele or demethylation of the *KvDMR1* locus has been found in Wilms tumors and a subset of BWS patients displaying loss of *IGF2* imprinting, suggesting that the relaxation of *IGF2* imprinting may be caused by an epigenetic alteration (Moulton et al. 1994; Steenman et al. 1994; Taniguchi et al. 1995; Reik and Maher 1997; Smilinich et al. 1999). Consistent with this hypothesis, activation of the maternal *IGF2* allele was achieved by perturbation of DNA methylation or histone acetylation with specific inhibitors in animals or cultured cells (Hu et al. 1997, 1998; Pedone et al. 1999). It has been proposed that the demethylation of the *KvDMR1* locus is independent of *IGF2* imprinting but is linked to the imprinting of the *LIT1* (or *KvLQT1*) gene and possibly to the expression of other 11p15.5 genes involved in the pathogenesis of BWS (Lee et al. 1999). Our results are consistent with this hypothesis, since they show that the relaxation of *IGF2* imprinting can occur in the absence of altered methylation at both *H19* and *KvDMR1* loci. Our results also support the notion that *KvDMR1* demethylation is a BWS-specific epigenetic lesion.

Very few molecular data are available on KTWS. In a single case, a translocation with a breakpoint in the 11p15.1 region was found, raising the question of a putative gene located on chromosome 11p involved in the pathogenesis of this disease (Whelan et al. 1995). Rare familial aggregation of KTWS cases has been reported, and paradominant transmission was proposed (Aelvoet et al. 1992; Happle 1993). This mechanism would imply that a predisposing single-gene defect is

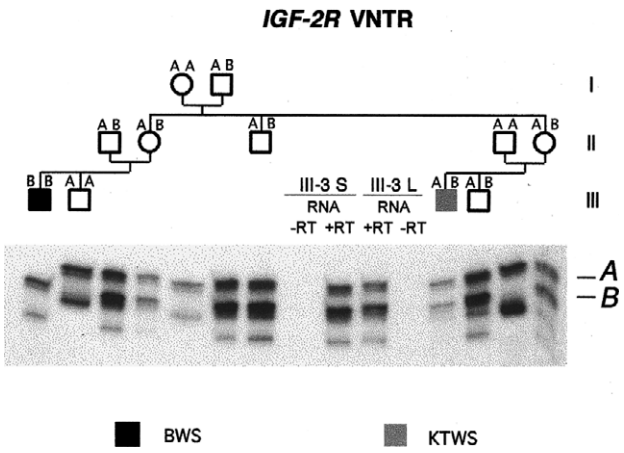

Figure 4 Genotypes of the family analyzed for the *IGF2R* VNTR marker and allele-specific expression of the *IGF2R* gene in the patient with KTWS. *IGF2R* alleles were analyzed by PCR and RT-PCR in DNA extracted from blood leukocytes and in RNA derived from cultured skin fibroblasts of the KTWS patient (III-3) by primers specific for the *IGF2R* VNTR polymorphism. Fibroblasts derived from both the larger (L) and smaller (S) sides of the body were analyzed. Two alleles, indicated as A and B, were observed. The "shadow" bands of the A and B alleles are an artifact commonly observed when dinucleotide-repeat polymorphisms are typed and are very likely due to slipped-strand mispairing during PCR amplification (Haugé and Litt 1993).

dominantly inherited and that the disease develops only after occurrence of a second somatic mutation. The finding of relaxation of *IGF2* imprinting suggests for the first time that overexpression of this growth-factor gene may be involved in the pathogenesis of this overgrowth syndrome.

To our knowledge, there are no previous reports of BWS and KTWS phenotypes in the same pedigree. In the family we studied, BWS and KTWS may represent the result of a clinical spectrum of somatic overgrowth caused by partially overlapping epigenetic alterations, ranging from relaxation of *IGF2* imprinting to disruption of both the imprinted *IGF2* expression and *KvDMR1* methylation. Clinical features typical of BWS may be secondary to demethylation of *KvDMR1*. Although we cannot exclude the possibility that the BWS and KTWS cases described were sporadic, the results are more likely explained by the maternal inheritance of a defective modifier or regulatory gene not linked to 11p15.5, which disrupted *IGF2* imprinting in the germ line or the early development of the two cousins and possibly also caused the *KvDMR1* demethylation in the BWS proband. The present report, together with the reports of the relaxation of *IGF2* imprinting found in several patients with BWS and four reported instances of nonsyndromic somatic overgrowth, reinforces the hypothesis of a general role of *IGF2* overexpression in various human diseases having tissue overgrowth as a common feature (Morison et al. 1996).

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Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim (for BWS [MIM 130650] and KTWS [MIM 149000])

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