

MOLECULAR ANALYSIS OF A PATIENT WITH
BECKWITH-WIEDEMANN SYNDROME,
RHABDOMYOSARCOMA AND RENAL
CELL CARCINOMA

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Summary We described a patient with Beckwith-Wiedemann syndrome associated with rhabdomyosarcoma (RMS), and renal cell carcinoma (RCC). Karyotypes of peripheral lymphocytes and RMS cells were normal. DNA analyses showed maternal loss of heterozygosity (LOH) at 11p15 region in RMS but not in RCC. The insulin-like growth factor II gene (*IGF2*) was found to be expressed at a moderate level in RMS but not in RCC by *in situ* hybridization. Each of parental allele-derived *IGF2* transcript was detected in RCC, while maternal allele-derived transcript was weak in RMS because of maternal LOH. These results suggest that (1) loss of imprinting (LOI) of *IGF2* might be responsible for BWS, (2) on the other hand, LOI itself might not induce tumor occurrence in tissues where the control of tissue-specific expression of *IGF2* is maintained, (3) increased expression of *IGF2* due to maternal loss of a putative controller gene for *IGF2* at 11p15 might predispose to sustaining tumorigenic mutations and tumor progression, (4) loss of a putative onco-suppressor gene at 11p15 might induce RMS occurrence. The cause of RCC was thought to be different from that of RMS.

Key Words Beckwith-Wiedemann syndrome, rhabdomyosarcoma, renal cell carcinoma, insulin-like growth factor II gene, loss of heterozygosity, loss of imprinting

INTRODUCTION

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth syndrome, which is sometimes associated with embryonal tumors, such as Wilms tumor, rhabdomyosarcoma (RMS), hepatoblastoma and adrenal carcinoma (Cohen, 1989). The BWS locus is localized to 11p15, where the genes for insulin (*INS*) and insulin-like growth factor II (*IGF2*) are also mapped; the latter might be responsible for perinatal gigantism and visceromegaly in BWS. In sporadic cases of BWS, paternal uniparental disomy or partial trisomy of paternal origin involving an 11p15 region have been known (Henry *et al.*, 1991; Mannens, 1991), while the predominance of maternal transmission of the disease in familial BWS cases have been reported (Niikawa *et al.*, 1986). Moreover, there is evidence that *IGF2* is maternally imprinted (paternally expressed) in both the mouse and the human (Rainier *et al.*, 1993). Therefore, a role of *IGF2* in a cause of BWS and/or tumor occurrence or progression is highlighted.

Here, we describe a BWS patient who developed in a short period rhabdomyosarcoma and renal cell carcinoma, together with the results of cytogenetic and molecular analyses which confirmed loss of heterozygosity (LOH) for a part of 11p15 in the RMS cells.

MATERIALS AND METHODS

Patient. The patient is a 3-year-old boy. He was born at the 37th week of gestation to non-consanguineous parents with a birth weight of 3,430 g (+1.3 SD). He suffered from tachypnea and feeding difficulty on day 1 and admitted to our hospital. He had a macroglossia, diastasis recti, hepatomegaly and hypoglycemia (20–30 mg/dl). Ear lobes were of normal appearance. He was diagnosed as BWS. At age 22 months, a lower abdominal mass was found and a needle biopsy revealed an embryonal type of rhabdomyosarcoma. The patient was treated according to the Intergroup Rhabdomyosarcoma Study (IRS) III protocol, then the tumor mass was subtotally resected, and irradiation was done to the peritoneal region during the surgical operation. At age 37 months during the following chemotherapy, another tumor was detected in the right kidney by computed tomography. The second tumor was totally removed and the same chemotherapy regimen has been going on. Pathological examination revealed that the second tumor was not a Wilms tumor but a renal cell carcinoma (RCC).

Chromosome analysis. Chromosome analyses with conventional and high resolution GTG-banding were done on cultured peripheral blood lymphocytes. As for RMS, a resected fresh tissue was minced with scissors and treated with a solution containing 0.8% collagenase and 0.002% DNase I for 15 min. The cell pellet was washed with PBS and cultured for 10 days with the ES medium (Nissui)

supplemented with 20% FBS. G-banding chromosome analysis was performed in 20 metaphase cells. RCC tissue was not available for karyotype analysis.

Molecular analysis. DNA from peripheral blood leukocytes (PB) of patient and his parents and from the RMS tissue was digested with several endonucleases and analyzed with Southern hybridization. Probes used were genomic DNA for the tyrosine hydroxylase gene (*TH*), *INS*, and *IGF2* (kindly given by Dr. G. I. Bell), *c-H-ras* (from JCRB) and *D11S12* (kindly given by Dr. Y. Nakamura). Another part of DNA was used for a polymerase chain reaction (PCR) assay to detect microsatellite nucleotide repeat polymorphism and restriction fragment length polymorphisms (RFLPs) for *TH* (Human Gene Mapping 11, 1991), *IGF2* (Ogawa *et al.*, 1993a), *H19* (Rainier *et al.*, 1993; Matsumoto *et al.*, 1994a), the Wilms tumor suppressor gene (*WT1*) (Zhang and Tycko, 1992; Matsumoto *et al.*, 1994b) and *D11S527* (Human Gene Mapping 11, 1991). Each primer was synthesized by Gene Assembler Plus (Pharmacia LKB). PCR was done as described previously (Kinoshita *et al.*, 1991). Single strand conformation polymorphism (SSCP) analysis was also done for *WT1*, *H19*, and *INS*. Reverse transcriptase-based PCR (RT-PCR) was done for *IGF2* on RNA extracted from RMS, RCC, and PB. These products were excised and eluted out from gel, then an aliquot was used for endonuclease digestion and nested PCR.

Messenger RNA in situ hybridization. A 534 bp *IGF2* cDNA fragment subcloned into pGEM-3 was labeled with digoxigenin-UTP and was used as sense and anti-sense RNA probes. Sample preparation and hybridization were done as described previously (Yun, 1993).

RESULTS

Chromosome analysis

Both peripheral blood lymphocytes and RMS cells showed a normal 46,XY karyotype.

Southern blot analysis

Loss of heterozygosity (LOH) was observed at the *TH* locus in the RMS cells (Fig. 1); maternally-derived 2.8 and 2.0 kb fragments observed in leukocytes were absent in the RMS tissue. Southern analysis also showed maternal LOH in the *IGF2* and *INS* loci (data not shown), while the other two loci (*c-H-ras* and *D11S12*) were not informative for polymorphisms in this family.

PCR analysis

Informative data were obtained in an RFLP at the *IGF2* locus, a tetranucleotide repeat polymorphism (TNRP) at the *TH* locus and a dinucleotide repeat polymorphism (DNRP) at the *D11S527* locus. The *ApaI*-digest of *IGF2*/PCR product (primers, P2/P3) showed a homozygous pattern for the 231 bp/61 bp allele in the

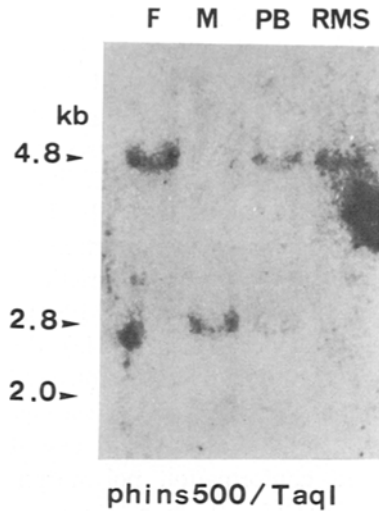


Fig. 1. Southern blot analysis of the pedigree including tumor tissues. DNA (10 μ g) was digested with *TaqI* and hybridized with the tyrosine hydroxylase gene genomic probe, phins500. Father (F) was homozygous for a 4.8 kb fragment, mother (M) homozygous for 2.8 kb/2.0 kb bands, and the patient's leukocytes (PB) heterozygous for both alleles, while the rhabdomyosarcoma cell (RMS) was hemizygous for the 4.8 kb fragment.

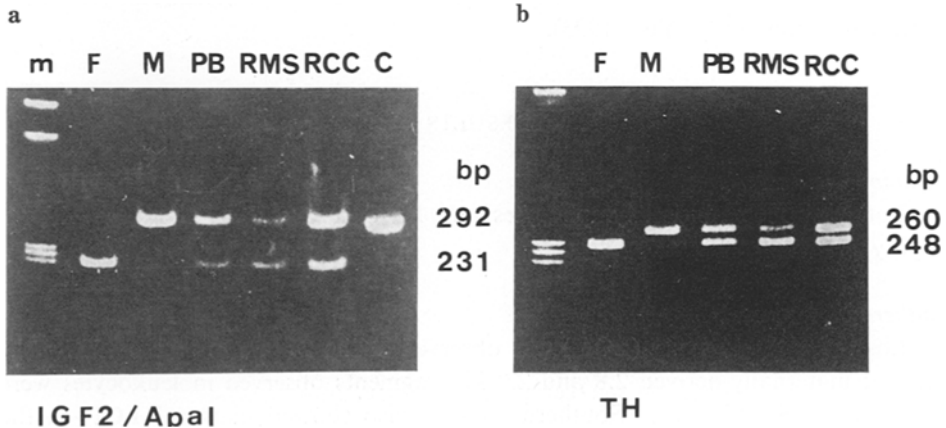


Fig. 2. *ApaI* digested *IGF2* PCR products (a). PCR products were digested with *ApaI*, separated on 6% polyacrylamide gel and stained with ethidium bromide (EtBr). Father (F) and mother (M) are homozygous for a 231 bp allele and for a 292 bp allele, respectively. Patient's peripheral leukocytes (PB), rhabdomyosarcoma (RMS), and renal tumor (RCC) are all heterozygous for both alleles, but the band intensity of the maternal allele in RMS is weak compared to that of PB or RCC. C, control, non-digested *IGF2* product. Left lane shows a size marker. PCR products of TH (b). PCR products were electrophoresed on 6% acrylamide gel and stained with EtBr. The band intensity of the maternal allele in RMS is reduced as in (a).

Table 1. Gene order and heterozygosity in the BWS patient.

Tissue examined	Gene order (pter-cen-qter)							
	<i>HRAS1</i>	<i>TH</i>	<i>INS</i>	<i>IGF2</i>	<i>H19</i>	<i>D11S12</i>	<i>WT1</i>	<i>D11S527</i>
PB	NI	H	H	H	NI	NI	NI	H
RMS		LOH	LOH	LOH				H
RCC		nd	nd	H				H

PB, peripheral blood; Rh, rhabdomyosarcoma; RT, renal tumor; NI, not informative; H, heterozygous; LOH, loss of heterozygosity; nd, not done.

father, a homozygous pattern for a 292 bp allele in the mother, and a heterozygous pattern for the two alleles in PB and RCC of the patient. In the RMS tissues, the intensity for the 292 bp maternally-derived allele was much low, compared with those in PB and RCC (Fig. 2a). Similar findings were obtained in *TH*-TNRP (Fig. 2b). At the *D11S527* locus, the PCR products from each of the two tumor tissues showed an equal intensity between the maternal and paternal alleles (data not shown). Other polymorphisms, *WT1/HinfI* RFLP, *WT1/3'* dinucleotide (CA) repeat polymorphism, *H19/RsaI*, *AluI*, and *HhaI* RFLPs, *H19*-, *WT1*-, and *INS*-SSCPs, were all uninformative in this family. The results of Southern blot and PCR analyses were summarized in Table 1.

The *IGF2* RT-PCR product was digested with *PstI* or *ApaI*. *PstI* digests of 1,123 bp *IGF2*/RT-PCR products (primers, P1/P3) from each of RMS and RCC tissues gave 851, 351, and 21 bp fragments which coincided with the restriction map of the *IGF2* cDNA. *ApaI* digestion revealed 892 and 231 bp fragments and a very weak 1,123 bp fragment from RMS. By nested PCR with primers P2/P3, 292 bp fragments were amplified from both of the tumors. *ApaI* digests gave 292, 231, and 61 bp fragments from RCC, while 292 bp fragment from RMS was very weak in intensity (Fig. 3, a, b, and c). PB of the patient also revealed both of parental allele-derived transcripts (Fig. 3c).

mRNA in situ hybridization

The *IGF2* transcript was detected in moderate levels in the cytoplasm of the RMS cells, while the transcript was almost absent in the co-existing fibrous tissue of RMS and RCC and the surrounding normal renal tissue (Fig. 4).

DISCUSSION

The frequency of complication of embryonal tumors is high in BWS patients, and rhabdomyosarcoma is the one of the associated tumors. It has been known that such an embryonal tumor tissue often shows LOH that involves loss of onco-suppressor gene(s). Southern blot and PCR analyses in the RMS from our BWS

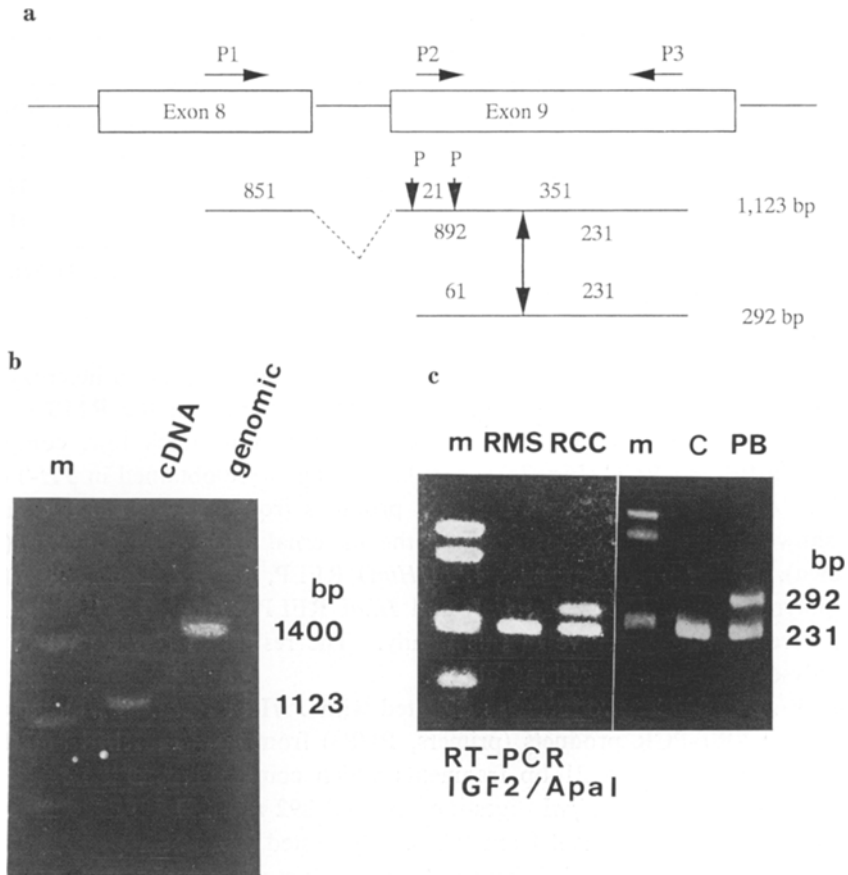
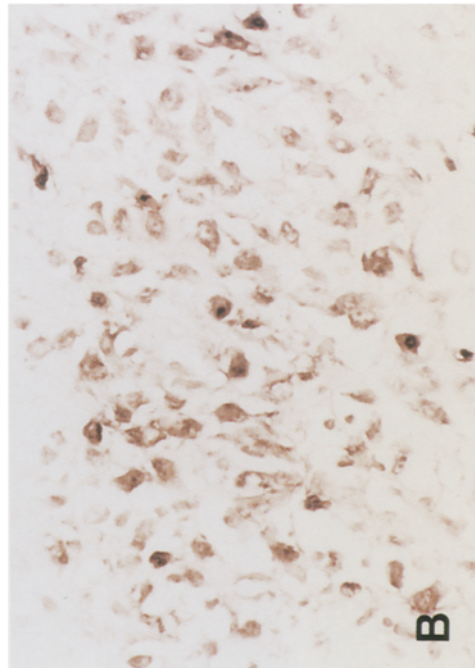
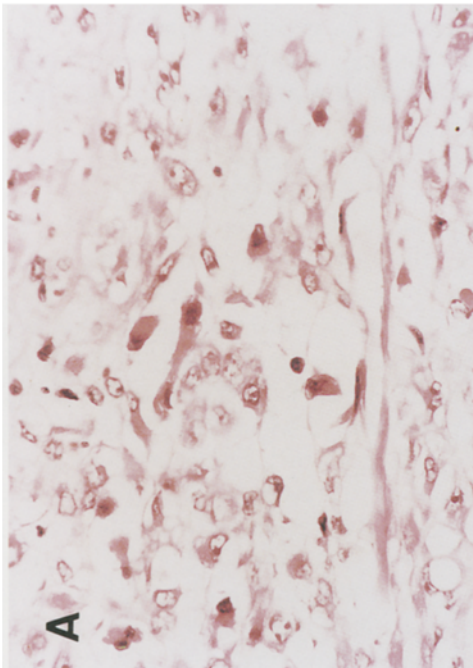
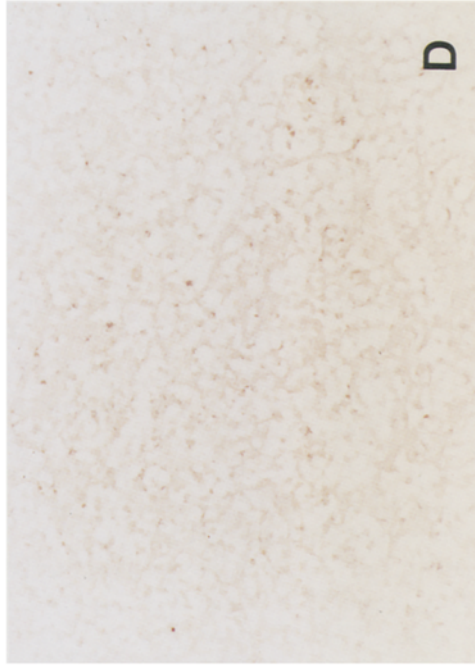
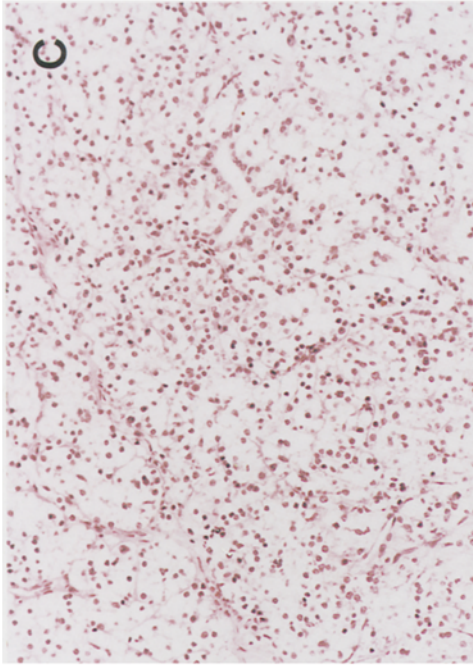


Fig. 3. *IGF2* RT-PCR products from rhabdomyosarcoma cells. a) *IGF2* gene map and sites of primers (P1–3) (a). P indicates *PstI* sites. Bi-directional arrow shows a polymorphic *ApaI* site. RT-PCR and genomic PCR products from RMS tissue using primers P1 and P3 (b). c) P1/P3 RT-PCR products from RMS, RCC, and PB underwent nested PCR using primers P2/P3, then the products were digested by *ApaI* (c). RCC and PB showed both of parental allele-derived transcripts, while maternal allele-derived transcript in RMS was weak in intensity. C, peripheral blood from normal control; m, size marker.

Fig. 4. mRNA *in situ* hybridization with *IGF2*. A high magnification of embryonal rhabdomyosarcoma (original magnification, $\times 450$, hematoxylin-eosine) (A). *In situ* hybridization of RMS in which virtually all tumor cells contain abundant *IGF2* transcripts (original magnification, $\times 450$, no counterstaining) (B). Renal clear cell carcinoma (original magnification, $\times 225$, hematoxylin-eosine) (C). *In situ* hybridization of RCC in which no *IGF2* transcripts were detected (original magnification, $\times 225$, no counterstaining) (D).



patient showed maternal LOH at the *TH*, *INS*, and *IGF2* loci all located to 11p15.5. As *DIIS12* at 11p15.5 and *WT1* at 11p13 were not informative in this family, we could not estimate a deletion extent on the chromosome 11 in the tumor cells. It seems less likely that the extent was large, because in the tumor tissue on visible deletion was seen by chromosome analysis.

IGF2 expression is controlled by tissue- and development-specific ways. *IGF2* has four different promoters and the use of each promoter seems to depend on different tissue-specific transcription factors (Sussenbach *et al.*, 1992). *IGF2* is known to be maternally imprinted: only the paternally-derived allele is active, while the maternally-derived one is inactive during embryogenesis and also in one BWS patient (Ohlsson *et al.*, 1993). Recently, bi-allelic expression of *H19* and *IGF2*, namely, relaxation of imprinting or loss of imprinting (LOI) has been reported in Wilms tumor tissues, and the LOI may be causally related to tumorigenesis (Ogawa *et al.*, 1993a; Rainier *et al.*, 1993). Because of LOH at 11p15 in RMS of our patient, the tumor cell itself might express *IGF2* only from paternally-derived allele, while both of parental alleles of *IGF2* were transcribed in RCC, leukocytes and probably in normal fibrous tissue of RMS. These results suggest that LOI might occur in the whole body in our patient and result in the occurrence of BWS. Very recently, LOI has been reported in some tissues including Wilms tumor of a patient with gigantism (Ogawa *et al.*, 1993b) and in 4 of 6 BWS fibroblast strains (Weksberg *et al.*, 1993). Therefore, bi-allelic expression of *IGF2* due to a mutation of a putative *cis*- or *trans*-acting element on maternally transmitted chromosome 11 which induces imprinting of *IGF2*, or paternal disomy of 11p15 might induce overexpression of *IGF2* resulting in BWS. In this context, *H19* is very interesting, because it is located near the *IGF2* locus (Zemel *et al.*, 1992) and known to be imprinted inversely to *IGF2* (Zhang and Tycko, 1992). It might be associated with *IGF2* imprinting, because it may function as the nuclear RNA (Brannan *et al.*, 1990), as in case of *XIST* on X chromosome inactivation (Brown *et al.*, 1991). We could not elucidate the imprinting status of the *H19* gene in either of the tumor tissues, since any *H19* polymorphisms were uninformative in our family.

In situ hybridization revealed that the *IGF2* transcript in the tissue level was moderate in RMS cells but almost nil in RCC and the fibrous tissue of RMS, although both parental *IGF2* alleles were expressed on RT-PCR analysis. Renal cell carcinoma is derived from the renal epithelium in which *IGF2* expression is absent even in the fetal kidney (Yun, 1993). These data suggest that bi-allelic expression of *IGF2* itself might not mean increased expression of *IGF2*, nor induce tumor occurrence and/or progression, especially in tissues where *IGF2* is not usually expressed and/or when *IGF2* expression is developmentally regressed. The increased expression of *IGF2* in some tissues may influence the growth of tumor(s). It is known that *IGF2* expression is increased in some tumor tissues including embryonal tumors (Sussenbach *et al.*, 1992). In some tumors involving LOH at 11p15, loss of the function of a putative onco-suppressor gene at 11p15 (Koi *et al.*, 1993) might induce the tumor occurrence and progression. Therefore, the constitutional bi-

allelic expression of *IGF2* may cause somatic overgrowth, which predisposes to tumorigenic mutations (Chao *et al.*, 1993) and/or rapid growth of tumor by the *IGF2* autocrine mechanism.

Our patient developed two different neoplasms, RMS and RCC, in a short period. These two tumors might have occurred through different mechanisms of tumorigenesis, because the LOH of chromosome 11p was seen in RMS but not in RCC. The former was likely associated to BWS, whereas a mechanism of the occurrence of the latter remained unknown but could be associated to irradiation and/or chemotherapy as in the case of Banner *et al.* (1977) or to other gene abnormality such as a putative onco-suppressor gene on chromosome 3p (Knudson, 1993).

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