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Tumor-specific loss of 11p15.5 alleles in del11p13 Wilms tumor and in familial adrenocortical carcinoma

(mapping/cancer/*WAGR* locus)

I. HENRY*, S. GRANDJOUAN†, P. COUILLIN*, F. BARICHARD*, C. HUERRE-JEANPIERRE*, T. GLASER‡, T. PHILIP§, G. LENOIR¶, J. L. CHAUSSAIN‖, AND C. JUNIEN*,**

*Institut National de la Santé et de la Recherche Médicale Unité 73, Château de Longchamp, Bois de Boulogne, 75016 Paris, France; †Service de Gastro-entérologie, Hôpital Cochin, 75014 Paris, France; ‡Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; §Service d'oncologie pédiatrique, Centre Léon-Bérard, 69008 Lyon, France; ¶Division des carcinogènes de l'environnement, International Agency for Research on Cancer, 69372 Lyon, France; and ‖Service d'Endocrinologie, Hôpital Saint Vincent de Paul, 75014 Paris, France

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ABSTRACT **We have compared constitutional and tumor genotypes in nine cases of hereditary Wilms tumor (WT) and in three unrelated cases of familial adrenocortical carcinoma (ADCC). Since susceptibility to these tumors can be observed in malformation syndromes associated with a constitutional deletion of band 11p13 (WT) and with a constitutional duplication of band 11p15.5 (WT, ADCC), we investigated these two candidate regions by using 11p polymorphic markers. As expected, somatic chromosomal events, resulting in a loss of heterozygosity limited to region 11p15.5, were observed in the tumor of two familial cases of adrenocortical carcinoma. Surprisingly, however, analysis of the WT of two patients with a constitutional deletion of band 11p13, associated with aniridia, genitourinary abnormalities, and mental retardation (WAGR syndrome), revealed a loss of heterozygosity limited to region 11p15.5. These data therefore suggest that observation of a specific loss of heterozygosity may not necessarily point to the site of the initial germinal mutation. Together with previous similar observations of a loss of heterozygosity limited to 11p15.5 in breast cancer and in rhabdomyosarcoma, our data suggest that region 11p15.5 may carry a non-tissue-specific gene that could be involved in genetic predisposition, in tumor progression, or in both.**

As is widely acknowledged, inheritance plays a role in the development of human cancers. Bilateral tumors, multicentric tumors, or multiple primary malignancies in the same individual and/or in a first-degree relative may indicate increased susceptibility to malignancy as a result of predisposing host factors (1–3). Hereditary tumors are thought to arise after a primary germinal mutation is followed by a somatic mutation affecting the same gene on the homologous chromosome (1, 4).

In the case of other childhood tumors—namely, Wilms tumor (WT), adrenocortical carcinoma (ADCC), hepatoblastoma, and rhabdomyosarcoma—hereditary predisposition is observed in association with several malformation syndromes that seem to involve genes located in different areas of chromosome 11 and possibly in other as yet unidentified regions. (*i*) The WAGR syndrome is a complex syndrome that associates a predisposition to WT, aniridia (A), genitourinary abnormalities (G), mental retardation (R), and a constitutional deletion of band 11p13 (5). (*ii*) The Beckwith–Wiedemann syndrome (BWS) is a polymalformation syndrome associating a predisposition to several types of tumors, including WT (59% of the tumors), ADCC (15%), hepatoblastoma, and rhabdomyosarcoma (6). Although ADCC is a rare childhood tumor (0.4% of malignant tumors), it occurs with an increased frequency in patients with BWS (40-fold) (7). ADCC is also more frequently (12% versus 1%) associated in the same individual or in the same family with other tumors that belong to the same type as those observed in BWS (8). Most cases of BWS are sporadic; however, a constitutional duplication of region 11p15 has been reported in 12 cases (9). (*iii*) The Drash syndrome is known to be often complicated by WT and, to date, no chromosomal abnormality has been described (10). (*iv*) Hemihypertrophy is often observed in children with WT or ADCC (8).

Although specific losses of chromosome 11 alleles have been extensively demonstrated in WT (11–14), in rhabdomyosarcoma, and in hepatoblastoma (15), rigorous proof that the chromosome remaining in the tumor carries the original predisposing mutation has not been presented. In ADCC, there has been no report, to date, of any specific loss of heterozygosity in such tumors.

To test the two-hit model of Knudson and Strong (16), we postulated that WT in WAGR patients should arise after a second mutation occurred in 11p13, whereas WT in BWS patients should arise following a somatic mutation in 11p15. In other cases without formal indication of the site of the germinal mutation—namely, in bilateral cases and in Drash syndrome—11p13, 11p15, or another region (17, 18) might be involved. We also tested the same hypothesis—i.e., a somatic mutation in 11p15 for ADCC. We based this test on clues as to the location of a gene for susceptibility to ADCC provided by the molecular definition of a constitutional duplication of 11p15.5 in a BWS patient with ADCC and by the increased frequency of ADCC in patients with BWS (7, 19, 20).

Genotypes were determined in normal and tumor tissues of different etiologies from nine cases of hereditary WT, including del11p13 WAGR syndrome, BW syndrome, Drash syndrome, and bilateral cases. Three unrelated cases of hereditary ADCC were examined for loss of alleles in region 11p15.5.

MATERIAL AND METHODS

Patients. All patients studied are listed in Table 1. Patients BAN, DAU, and ZER presented with WAGR syndrome, nephroblastoma, and del11p13→11p12 (patients BAN and DAU) or del11p14→11p12 (patient ZER). Patients POI, REI, and KIA presented with BWS and nephroblastoma. Patient AUB presented with Drash syndrome and nephroblastoma. Patient ANS was a bilateral case of nephroblastoma. Patient FIR was a familial case of nephroblastoma.

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Abbreviations: WT, Wilms tumor; ADCC, adrenocortical carcinoma; BWS, Beckwith–Wiedemann syndrome; WAGR, predisposition to WT, aniridia, genitourinary abnormalities, and mental retardation; RFLP, restriction fragment length polymorphism.

**To whom reprint requests should be addressed.

The pedigrees of the three unrelated cases of familial ADCC studied are shown in Fig. 1. In family HAM, a tumor sample was obtained from only one of the affected brothers, HAM 5. As shown in Fig. 1, in family ZEM and family BOL, there was evidence for familial predisposition to several types of cancer, including osteosarcoma, breast cancer, ADCC, and rhabdomyosarcoma. All cases of ADCC studied here presented with signs of virilism. Karyotype analysis failed to reveal a chromosomal rearrangement in all of these latter patients (data not shown).

Somatic Cell hybrids. Somatic cell hybrids were established by fusing a lymphoblastoid cell line from patients BAN and DAU and a mouse cell line Sp2/0-Ag14, as described (21).

Probes. Southern blots were hybridized to the following probes that detect restriction fragment length polymorphisms (RFLPs) on 11p (22): *HRAS1* (c-Ha-*ras* 1); *INS* (insulin); *IGF2* (insulin-like growth factor II); *D11S12* (pADJ762); *HBB* (β-globin); *CALCA* (calcitonin 1); *PTH* (parathyroid hormone); *FSHB* (follicle-stimulating hormone subunit β); *CAT* (catalase); *D11S9* (pES1-2); *APOAI* (apolipoprotein A1); *ETS1* (c-ets 1). *COL2A1* (collagen α1 type 2) (23) and *APOC2* (apolipoprotein C2) (24) were used as non-11 internal controls. 33.15 (minisatellite) is a *Pst* I/*Ava* II genomic fragment that detects randomly distributed multiallelic loci (25).

Southern Blot Experiments. DNA was prepared from lymphocytes, fibroblasts, or lymphoblastoid cell lines as described (26). Aliquots of 15–30 μg of DNA were digested with restriction endonucleases according to the manufacturer's recommendations. Restriction endonucleases were purchased from Promega Biotec, [^{32}P]dCTP and the nick-translation kit were from Amersham. DNA was electrophoresed and then transferred to nitrocellulose or nylon membrane as described by Southern (27). Hybridization with ^{32}P-labeled probes (specific activity, 2–6 $\times$ 10^8 cpm/μg) was carried out for 16 hr using 10% dextran sulfate as described (28). For gene copy number determination, the filters were freed of probe in alkali and were rehybridized with other probes used as internal control. The intensity of the hybridization signals was measured with a SEBIA densitometer, and the ratio of each test 11p probe versus control probe signal intensity was calculated. Each experiment was performed in triplicate. Each result was subsequently normalized to the control ratio (1.00). Statistical analysis (Student's *t* test) was performed using independent values obtained for the normal control and for constitutional and tumor DNA from the patients.

RESULTS

To define which of the 11p regions, 11p13 or 11p15, was involved in the tumors, the constitutional DNAs were analyzed with eleven probes detecting 25 RFLPs spanning chromosome 11 from pter to qter. Tumor DNAs were subsequently analyzed with probes that detected heterozygosity at the constitutional level. Analysis of constitutional and tumor informative pairs showed a loss or marked significant reduction in intensity of one of the two alleles in 5 of 12 tumors (Table 1).

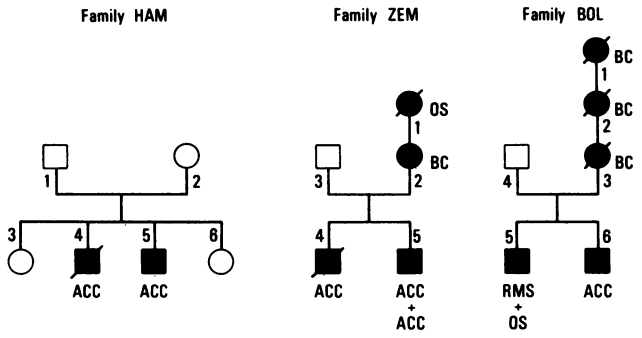

FIG. 1. Pedigrees of families with ADCC (ACC). Other tumors observed were osteosarcoma (OS), breast cancer (BC), and rhabdomyosarcoma (RMS).

Constitutional Deletion of 11p13 in WAGR Patients. Patients BAN, DAU, and ZER were typical WAGR patients with 11p13 deletions. Gene copy number determination performed on constitutional DNA for *CAT* and *FSHB* using *COL2A1* as a non-11p internal control showed that all three patients have only one copy of these markers (Table 1). Moreover, hybrids established with BAN and DAU, which had retained the 11p− chromosome, confirmed the extent of the deletion in both cases and enabled us to determine the haplotypic phase in BAN (21).

Loss of 11p15.5 Alleles in Hereditary WT. Tumor DNAs showed losses of heterozygosity at polymorphic loci along the length of chromosome 11 (Table 1): BAN (losses at *HRAS1* and *CALCA*), ZER (losses at *HRAS1*, *D11S12*, and *HBB*), ANS (losses at *INS*, *HBB*, and *CALCA*). In contrast, the patients remained heterozygous or hemizygous, in their tumor for the following informative loci: BAN was hemizygous for *FSHB* and *CAT*; ZER was hemizygous for *FSHB* and *CAT* and heterozygous for *PTH*, *CALCA*, and *APOA1*; and ANS was heterozygous for *D11S9*. The tumor of patient DAU was heterozygous or hemizygous at all informative loci examined (*FSHB*, *CAT*, *PTH*, *CALCA*, *D11S12*, and *HRAS1*) (Fig. 2*A*).

Karyotype analysis could not be performed on these tumors. Therefore, to determine whether these losses of heterozygosity were due to a somatic deletion, to a mitotic recombination, or to a more complex rearrangement, gene copy number determination was carried out for the markers flanking the region of the breakpoint in each tumor. In each experiment two different probes were used as internal controls. The first one was chosen in the region of chromosome 11, for which the patient remained heterozygous. The second one was a non-chromosome 11 sequence, for which the patient remained heterozygous in the tumor, used to compensate for unequal DNA loading in the different lanes. When a deletion could be demonstrated, the region of the breakpoint could thus be defined more precisely. As shown by densitometric scanning (Fig. 2*B*) in the tumor of patient BAN there was clearly only one copy for *CALCA* (ratio = 0.48) and two copies for *PTH* (ratio = 0.98), thus indicating a deletion with a breakpoint between *CALCA* and *PTH*. Moreover, according to the haplotypic phase determined using a hybrid that had retained the 11p− chromosome of patient BAN, we showed that the missing 11p15 region belonged to the normal chromosome 11 (21). In the tumor of patient ZER, there is an obvious difference of intensity (0.4) of the two alleles for *HRAS1*/*Bam*HI, *D11S12*/*Msp* I, and *HBB*/*Ava* II in the tumor. This is in contrast with what is observed for two more proximal markers—namely, *PTH* and *CALCA*—for which the patient remains heterozygous with no difference of intensities in the tumor by comparison with the constitutional pattern. This clearly indicates that a somatic event occurred between (*HRAS1–HBB*) and (*CALCA–PTH*). To determine whether these differences of intensities were due to either loss of the 2.2-kb allele for *HBB* and of the 7.6-kb allele for *HRAS1* or reduplication of the chromosome segment carrying the 2.0-kb allele for *HBB* and the 6.6-kb allele for *HRAS1*, we performed gene dosage analysis using a nonchromosome 11 probe, *APOC2*, for which the patient was heterozygous at the constitutional level (*Taq* I). Comparison of *HRAS1* alleles with the *APOC2* constant band on the same *Bam*HI blot clearly shows a marked diminution of the *HRAS1* number 1 allele. As often reported by other authors, contamination by normal diploid tissue may account for the presence of the other allele in lower amounts. Alternatively, this may suggest

Table 1. Losses of alleles

		tel		11p15.5						11p15.4				11p13				11p12		cen		11q	
		HRAS1		*INS*		*D11S12*		*HBB*		*CALCA*		*PTH*		*FSHB*		*CAT*		*D11S9*		*APOA1*		*ETS1*	
Patient	Method	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
WT																							
BAN	RFLP	1/2	1/−	—	—	—	—	—	—	1/2	1/−	—	—	1/0	1/0	1/0	1/0	—	—	—	—	—	—
	T/C									(0.48)		(0.98)											
DAU	RFLP	1/2	1/2	—	—	1/2	1/2	—	—	1/2	1/2	1/2	1/2	1/0	1/0	1/0	1/0	—	—	—	—	—	—
ZER	RFLP	1/2	*/2	—	—	1/2	*/2	1/2	*/2	1/2	1/2	1/2	1/2	1/0	1/0	1/0	1/0	—	—	1/2	1/2	—	—
	T/C	(0.69)				(0.67)		(0.71)															
POI	RFLP	1/2	1/2	—	—	1/2	1/2	1/2	1/2	—	—	1/2	1/2	—	—	1/2	1/2	—	—	1/2	1/2	—	—
REI	RFLP	—	—	—	—	—	—	—	—	—	—	1/2	1/2	—	—	—	—	—	—	1/2	1/2	—	—
KIA	RFLP	—	—	—	—	—	—	—	—	1/2	1/2	1/2	1/2	—	—	1/2	1/2	—	—	1/2	1/2	—	—
AUB	RFLP	1/2	1/2	—	—	—	—	—	—	—	—	1/2	1/2	—	—	1/2	1/2	—	—	—	—	1/2	1/2
ANS	RFLP	—	—	1/2	−/2	—	—	1/2	1/−	1/2	1/−	—	—	—	—	—	—	1/2	1/2	—	—	—	—
	T/C									(1.02)													
FIR	RFLP	—	—	1/2	1/2	1/2	1/2	—	—	—	—	—	—	—	—	—	—	—	—	1/2	1/2	—	—
ADCC																							
HAM	RFLP	—	—	—	—	1/2	−/2	—	—	1/2	1/2	—	—	1/2	1/2	—	—	1/2	1/2	—	—	—	—
	T/C					(1.12)		(1.2)															
ZEM	RFLP	—	—	—	—	—	—	1/2	−/2	—	—	1/2	1/2	—	—	—	—	—	—	1/2	1/2	—	—
	T/C									(0.55)		(1.00)											
BOL	RFLP	—	—	—	—	1/2	1/2	—	—	—	—	—	—	—	—	—	—	1/2	1/2	—	—	—	—

Shown is the allelic distribution for 11-specific DNA markers mapping from pter to qter in tumor DNA from nine cases of WT whether or not associated with malformation syndromes and from three hereditary cases of ADCC. Results are given only for loci informative at the constitutional level. The number of copies for *CAT* and *FSHB* was determined only in the constitutional DNA from the three patients with WAGR syndrome. The ratio was calculated by dividing the 11p/*COL1A2* ratio in the constitutional DNA from the patient by that in the DNA from normal individuals. RFLP alleles were named 1 and 2 according to decreasing length: 1/− or −/2 indicates the allele that remained in the tumor; 1/2 means that the tumor DNA remained heterozygous at this locus; */2 indicates that the tumor sample was slightly contaminated by diploid tissue (allele 1/allele 2 = 0.38); 1/0 means that the patient who carried a constitutional del11p13 retained the unique copy of this marker in its tumor cells. To determine the nature of the mitotic event that leads to loss of 11p15.5 alleles in patient BAN, ZER, HAM, and ZEM, gene copy number determination was performed. The ratio (T/C) was calculated by dividing the value found in the tumor DNA (T) by that in the constitutional DNA (C). The order of the markers is represented according to the last report of human gene mapping (18). tel, Telomere of chromosome 11; cen, centromere of chromosome 11.

that loss of the 11p15 allele corresponds to a progression event that is only represented in part of the tumor.

In the tumor of patient ANS there were two copies for *CALCA*, thus indicating a mitotic recombination between *CALCA* and *D11S9*. This patient was not informative for other markers mapping in this interval, thus not allowing us to define the exact breakpoint.

Loss of 11p15.5 Alleles in Hereditary ADCC. As shown in Fig. 2*A* and Table 1, loss of heterozygosity was found in tumor DNA from two of three hereditary cases: ZEM (loss at *HBB*) and HAM (loss at *D11S12*). The patients remained heterozygous in their tumor at the following loci: ZEM was heterozygous for *PTH* and *APOA1*, HAM was heterozygous for *CALCA* and *D11S9*, and BOL remained heterozygous at all informative loci examined (*HRAS1, INS, HBB, CALCA,* and *ETS1*). To determine whether these losses of heterozygosity were due to a mitotic recombination or to a deletion, we also performed gene copy number determination using internal controls: HAM displayed two identical copies for *D11S12* (ratio = 1.12) and two different alleles for *CALCA*, thus indicating a mitotic recombination between *D11S12* and *CALCA*. Since only one copy for *CALCA* (ratio = 0.55) was observed in the tumor of ZEM, the somatic event could be identified as a deletion with a breakpoint between *CALCA* and *PTH* (two different alleles).

Chromosome Specificity. To test whether the loss of heterozygosity in these hereditary cases of WT and ADCC was specific to chromosome 11 or part of a more general reduction to homozygosity, we hybridized the paired DNA samples from all patients with the minisatellite probe 33.15 that detects multiallelic RFLPs dispersed on the genome (25) (Fig. 3). We detected no evident difference between constitutional and tumor DNAs.

DISCUSSION

We have identified a chromosomal region, 11p15.5, that undergoes loss of constitutional heterozygosity during oncogenesis of hereditary WT and ADCC. According to the consensus gene order of the markers on 11p (29), this region extends from pter to *CALCA* excluded (19). Specificity of the region involved is crucial. However, it is difficult to determine whether the rearrangement observed is specific for each type of tumor in the absence of karyotype analysis and without RFLP analysis representative of the remainder of the genome. Nonetheless, several convergent types of data support our findings concerning region 11p15.5. (*i*) The same minimal region extending from pter to *CALCA/PTH* is involved in all of our informative patients as well as in rhabdomyosarcoma (30) and breast cancer (31). (*ii*) By using a minisatellite probe that detects several multiallelic loci dispersed on the genome, we found no evidence for other loss of alleles in the tumor DNAs digested by two different restriction enzymes.

In the WT/WAGR cases (BAN and ZER), the gene responsible for susceptibility to WT, and therefore the site of the germinal mutation, lies in 11p13 and is obviously different from that for which the loss of sequences was actually observed in the tumor cells—namely, 11p15.5. In patient ANS with bilateral WT, the site of the germinal mutation could not be determined in the absence of a visible cytogenetic abnormality and may therefore lie in 11p13, in 11p15, or elsewhere in the genome (17, 18). In the third WAGR patient, DAU, and in other cases of hereditary WT, using available 11p13 and 11p15 markers, we failed to detect a somatic loss of heterozygosity either in 11p13 or in 11p15.

As shown in two unrelated cases of ADCC (HAM and ZEM), the same region underwent a specific loss of alleles.

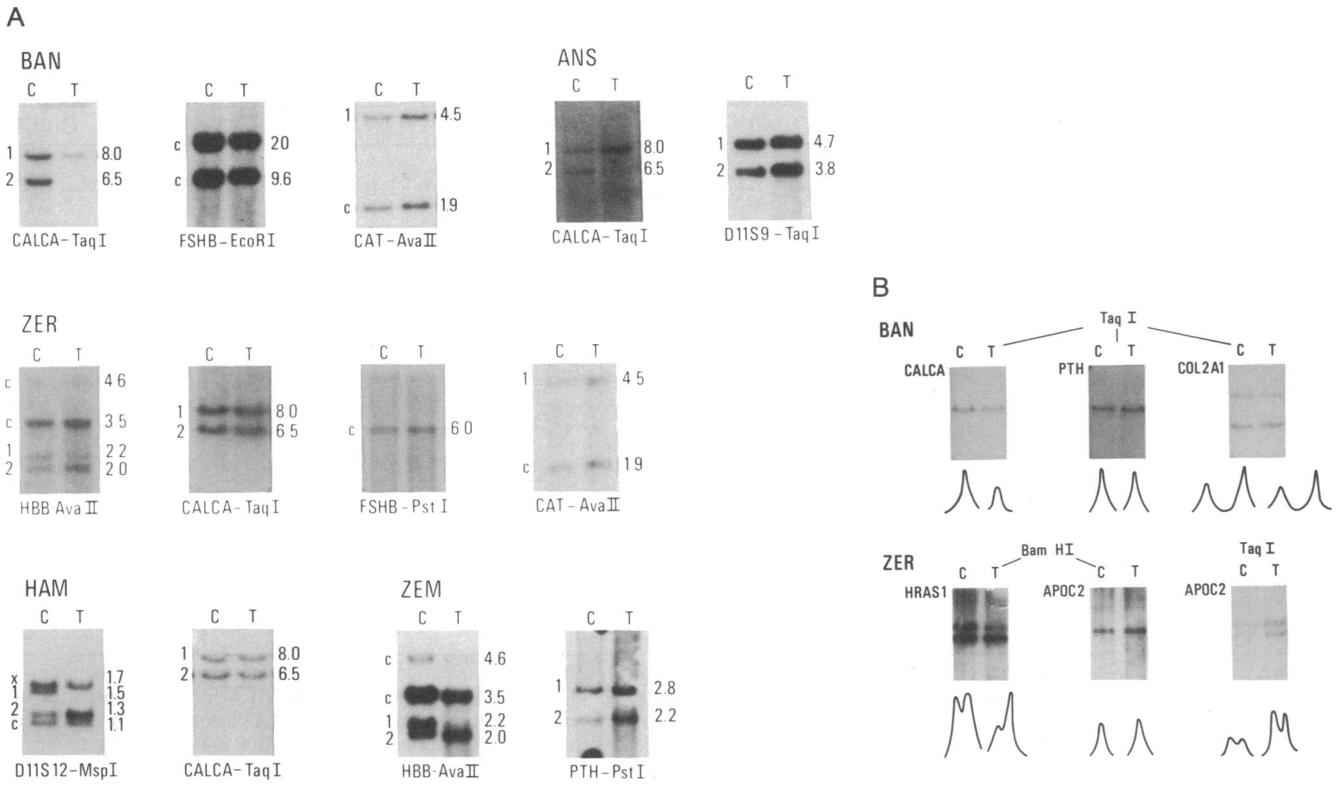

FIG. 2. RFLP analysis of loci on chromosome 11 in constitutional (C) and tumor (T) DNA from patients with WT and ADCC. (*A*) Representative Southern hybridization of specific 11p13 and 11p15 polymorphic markers flanking the breakpoints of the mitotic rearrangements observed in patients for whom a loss of heterozygosity was detected. The designation of alleles is to the left of each autoradiogram (c indicates a constant band), and allele lengths in kilobase pairs (kb) are to the right. x, Additional allelic system of *D11S12* for which patient HAM was not informative. (*B*) RFLP analysis and dosage analysis of loci on chromosome 11 in constitutional (C) and tumoral (T) DNA from patients BAN and ZER. The corresponding densitometer scannings are shown at the bottom of each blot. The same *Taq* I blot was hybridized with *CALCA*, *PTH*, and *COL2A1* for patient BAN. The same *Bam*HI blot was hybridized with *HRAS1* and *APOC2* for patient ZER. Patient ZER was heterozygous for *APOC2* (*Taq* I), with no difference in intensity between the two alleles, in the tumor by comparison with the constitutional pattern.

Based on our previous report of a BWS patient with ADCC (20) in whom we characterized a constitutional duplication also extending from pter to CALCA excluded (19), we can suggest that a gene for predisposition to ADCC lies in region 11p15.5. The observation in family ZEM and in family BOL of individuals with other types of cancer, including breast cancer and rhabdomyosarcoma for which a loss of heterozygosity limited to the same area of the genome has also been demonstrated (30, 31), is compatible with a hereditary mutation at a locus in 11p15.5. In contrast, the gene for predisposition to osteosarcoma, which is also observed both in family ZEM and in family BOL, has been shown to map to 13q14 (32), and recent data have shown loss of heterozygosity for 13q and rearrangement of the retinoblastoma gene in breast cancer (33). These apparently discordant observations could only be resolved by family studies with a sufficient number of informative meioses, which was not the case in the three families studied here.

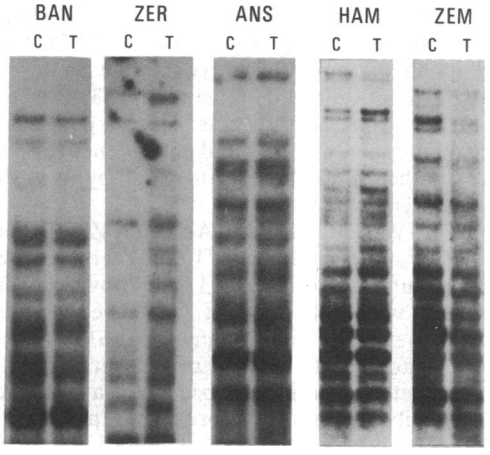

FIG. 3. DNA fingerprints for paired samples of constitutional (C) and tumoral (T) DNAs from patients BAN, ZER, ANS, HAM, and ZEM. DNA were digested by *Hin*fI and *Hae* III and hybridized to VNTR (variable number of tandem repeats) probe 33.15. Only one digest (*Hin*fI) per paired sample is shown here.

Region 11p15.5 thus contains a putative gene, or genes, clearly distinct from the 11p13 locus, which must be kidney-specific since tumors other than WT have never been reported in WAGR patients. Our data show that both loci may be involved in the same tumor, thus implying that the first one (on 11p13) is responsible for genetic predisposition, whereas the other one (on 11p15.5) is responsible for initiation or progression of the tumor. Family studies have shown recently in WT families that the gene for susceptibility to WT is not linked to 11p13 and 11p15 markers (17, 18). A loss of 11p15.5 allele was also shown in the tumor of one of the patients (18).

These observations therefore imply for hereditary predisposition to WT and probably for other cancers a more complex process than that of the two-hit mechanism at the same locus extensively documented for retinoblastoma (16, 34). Bearing in mind that Knudson's two hits represent a minimum estimate of the number of genetic events involved in tumorigenesis (1), we can propose that the germinal mutation is responsible for a latent or nonapparent alteration

of the cell functions or for the malformation syndrome itself (BWS, WAGR). Based on our observations in the WAGR patients, at least two different mechanisms can be suggested: (*i*) there are only two events, one germinal mutation in 11p13 and one somatic mutation in 11p15; (*ii*) there are three events, one germinal mutation in 11p13, one somatic mutation also in 11p13 (which could not be detected in our three patients using two 11p13 markers (*FSHB* and *CAT*), and one somatic mutation in 11p15. This is reminiscent of recent observations in multiple endocrine neoplasia type 2A (*MEN2A*) and in colon cancer, for which family studies have shown the presence of a gene involved in susceptibility to these cancers, on chromosome 10 and chromosome 5, respectively (35, 36), whereas losses of heterozygosity for markers mapping to other chromosomes, 1 for *MEN2A* (37) and 17 and 18 for colon cancer (38), were found in the same type of tumors.

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