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hSNF5/INI1 Inactivation Is Mainly Associated with Homozygous Deletions and Mitotic Recombinations in Rhabdoid Tumors[1]

Marie-Françoise Rousseau-Merck,[2] Isabella Versteege, Isabelle Legrand, Jérôme Couturier, Aline Mairal, Olivier Delattre, and Alain Aurias

Pathologie Moléculaire des cancers INSERM U509 [M-F. R-M., I. V., I. L., A. M., O. D., A. A.] and Unité de Cytogénétique Oncologique [J. C.], Institut Curie, 75248 Paris cedex 05, France

ABSTRACT

The chromatin-remodeling *hSNF5/INI1* gene has recently been shown to act as a tumor suppressor gene in rhabdoid tumors (RTs). In an attempt to further characterize the main chromosomal mechanisms involved in *hSNF5/INI1* inactivation in RTs, we report here the molecular cytogenetic data obtained in 12 cell lines harboring *hSNF5/INI1* mutations and/or deletions in relation to the molecular genetic analysis using polymorphic markers extended to both extremities of chromosome 22q. On the whole, mitotic recombination occurring in the proximal part of chromosome 22q, as demonstrated in five cases, and nondisjunction/duplication, highly suspected in two cases (processes leading respectively to partial or complete isodisomy), appear to be major mechanisms associated with *hSNF5/INI1* inactivation. Such isodisomy accompanies each of the RTs exhibiting two cytogenetically normal chromosomes 22. This results in homozygosity for the mutation at the *hSNF5/INI1* locus. An alternate mechanism accounting for *hSNF5/INI1* inactivation observed in these tumors is homozygous deletion in the rhabdoid consensus region. This was observed in each of the four tumors carrying a chromosome 22q abnormality and, in particular, in the three tumors with chromosomal translocations. Only one case of our series illustrates the mutation/deletion classical model proposed for the double-hit inactivation of a tumor suppressor gene.

INTRODUCTION

RTs[3] are highly malignant pediatric cancers. First described within the kidney as a rhabdomyosarcomatoid aggressive variant of Wilms' tumor (1), they were shown to arise in various sites such as brain and soft tissues (2). Extrarenal RTs are often difficult to distinguish from other poorly differentiated neoplasms, and their belonging to a single histological rhabdoid entity has been discussed (3–5). Despite their known aggressiveness, RTs are characterized by few or no visible karyotypic changes. Abnormalities such as partial deletions and translocations involving chromosome band 22q11.2 have been described in several RTs (6–8). These cytogenetic data prompted several molecular genetic studies to search for a probable tumor suppressor gene (8–10). Furthermore, the occurrence of familial cases (11) and coexistence of bifocal tumors within the same patient (12) fitted well with a RT suppressor gene context. Recently, the observation of biallelic alterations or deletions of *hSNF5/INI1* in 12 of 13 rhabdoid cell lines from tumors of different locations strongly suggested that this gene was the RT suppressor (13).

Allelic inactivation of a tumor suppressor gene has been proposed to occur through either point mutation or whole or partial deletion of a chromosome. Combination of these events can lead to the deletion/deletion, deletion/mutation, or mutation/mutation of both alleles of the gene. LOH at polymorphic loci appears as a common event associated with the expression of a recessive mutation (14); however, it is not always accompanied by a monosomic cytogenetic profile. Indeed, different chromosomal mechanisms such as nondisjunction/duplication or recombination at the G_2 phase of the cell cycle have been described to lead to uniparental disomy without any apparent karyotypic modification in retinoblastoma and Wilms' tumors (15–19). In these tumors, uniparental disomy has been demonstrated to result from the total or partial loss of one chromosome associated with the duplication of the remaining chromosome carrying the mutated allele.

In an attempt to further characterize the main chromosomal mechanisms involved in the *hSNF5/INI1* inactivation in RTs, we report here the molecular cytogenetic data obtained on 12 rhabdoid cell lines harboring *hSNF5/INI1* mutation or deletion (13) in relation to molecular genetic analyses using polymorphic markers extended to both extremities of chromosome 22q.

MATERIALS AND METHODS

Cell Lines

The 12 rhabdoid cell lines used in our series were obtained from tumors at different locations. All of them demonstrated either a homozygous deletion at the *hSNF5/INI1* locus (DL, TM87, LM, MON, G401, and KD) or a truncating mutation of one allele of the gene associated with the loss of the other allele (Wa2, LP, WT, MT, AS, and 2004; Ref. 13). These cell lines have been described previously (13), as follows: (*a*) 2004 (20); (*b*) TM87 (21); (*c*) Wa2 (22); (*d*) G401-ATCC (23); and (*e*) DL (8).

Constitutional material was obtained from blood or adjacent nontumoral tissues for six of these cell lines (DL, LP, MON, MT, WT, and 2004).

FISH Techniques

Chromosomes. Metaphase cells were obtained after incubation in 0.04 μg/ml Colcemid for 2 h at 37°C, followed by hypotonic treatment with 0.075 M KCl. The cells were fixed in methanol:acetic acid (3:1, v/v). The karyotype was analyzed by R or G banding.

DNA Probes. Two overlapping cosmids corresponding to the *hSNF5/INI1* locus, N96A6 and 77A2, were obtained from a chromosome 22 library (LL22NCO3). The YAC clone 792F9 was obtained from the Centre d'Etudes du Polymorphisme Humain YAC library. This YAC probe has been chosen in the centromeric part of the 22q11.2 region, next to the Di George locus, as a proximal marker. A telomeric probe of chromosome 22 (Telvision; Vysis) was used as a distal marker.

***In Situ* Hybridization.** Probes used in FISH analysis were labeled by nick translation with either digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) or biotin-14-dATP (Life Technologies, Inc.) and mixed with about 50-fold human Cot-1 DNA. Hybridization was performed as described previously (24). The slides were counterstained with 4′,6-diamidino-2-phenylindole and observed under a fluorescence Leica DMRB microscope. Images were acquired with an NU 200 charge-coupled device camera (Photometrics, Tucson, AZ) and analyzed with Smart Capture Software (Digital Scientific, Cambridge, England).

Microsatellite Analysis

To extend the limits of the previously characterized LOH regions (13), new microsatellite markers were selected based on the locations (three centromeric

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[2] To whom requests for reprints should be addressed, at INSERM U509, Institut Curie-section Recherche, 26 rue d'Ulm, 75248 Paris cedex 05, France.

[3] The abbreviations used are: RT, rhabdoid tumor; LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization; YAC, yeast artificial chromosome.

markers: D22S311, F8VWFP, and D22941; and five telomeric markers: D22S928, D221153, D22S1141, D22S1161, and D221169). The location and relative orders of these markers were determined from the chromosome 22 integrated map (25, 26).

DNA was extracted from cell lines, blood, or adjacent nontumor tissue according to Sambrook *et al.* (27).

Assessment of microsatellite polymorphisms was performed by PCR amplification in a final volume of 20 μl with 30 ng of genomic DNA, a mixture containing 1.5 mM $MgCl_2$, 100 mM each deoxynucleotide triphosphate, 0.3 mM each primer, and 0.4 μM Taq polymerase (Perkin-Elmer Cetus, Branchburg, NJ). The PCR conditions consisted of an initial denaturation at 96°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. PCR products were separated by electrophoresis on a 7 M urea/6% polyacrylamide gel in 15% Tris-borate/EDTA buffer. The gel was transferred to a nylon filter (Hybond N+) and hybridized with a (CA) 24-mer probe labeled with [α-^{32}P]CTP by using the terminal transferase kit (Boehringer Mannheim). Radiographs were exposed for 30 min to 2 h before development.

RESULTS

Karyotypes. Five cases (G401, LP, MT, AS, and 2004; Table 1) show a normal karyotype with a variable percentage of tetraploidy (from 3% to ≤50%).

Chromosome 22 abnormalities are observed in 5 of the 12 cases. Besides three cases carrying apparently balanced reciprocal translocations of 22q with different partners (chromosomes 1, 7, and 11 for DL, MON, and TM87, respectively), one case (Wa2) presents a 22p translocation with chromosome 18, and one case (LM) corresponds to a partial deletion of chromosome 22q without an identified reciprocal translocation.

Two cases demonstrate chromosomal abnormalities without the involvement of chromosome 22 (WT and KD).

In three cases (2004, KD, LM), the difference in the size of the short arms of two chromosomes 22 allows discrimination of one chromosome 22 from the other.

FISH. To exclude a possible misinterpretation of chromosome 22 during the FISH observations, we performed cohybridization of the 792F9 proximal probe labeled with digoxygenin and of the biotinylated *hSNF5/INI1* probes on the same metaphase preparation.

Two fluorescent signals specific to the 792F9 probe were obtained on both chromosomes 22 [normal or der(22)] in all metaphases from the 12 different cell lines (Table 1). In each case, a minor signal due to the chimeric status of the 792F9 probe was detected on chromosome 8.

Six cases (LP, WT, MT, AS, 2004, and KD) with two apparently normal chromosomes 22 gave positive signals on both chromosomes 22 after hybridization with the *hSNF5/INI1* probes. These signals could clearly be seen as two dots (or duplicated dots, depending on the G_1 or G_2 phase of the cell cycle) on the interphasic nuclei (Fig. 1*A*). They were observed in the KD cell line, in which the deletion involves only exons 4 and 5 of the gene. No signal could be detected on any other chromosome. One case (Wa2) carrying a translocation involving the short arm of chromosome 22 gave a signal with the *hSNF5/INI1* probe only on the normal chromosome 22. The der(22) chromosome involved in the t(18;22) translocation was negative (Fig. 1*B*). In five cases (DL, TM87, LM, MON, and G401), we did not detect any fluorescent signal corresponding to the *hSNF5/INI1* probes. This absence of signals on any chromosome or interphasic nuclei (Fig. 1*C*) confirms the homozygous deletion of both INI1 alleles on the two chromosomes observed previously at the molecular level (13). Interestingly, this absence of signal was observed in the four cases showing either a reciprocal translocation or a partial deletion involving 22q, demonstrating that these translocations are associated with submicroscopic deletions. Only G401 demonstrates a homozygous deletion associated with an apparently normal karyotype.

Hybridization with the telomeric probe of chromosome 22 showed two signals for all cases on the normal chromosome 22, and on either the second normal chromosome 22, the der(22), or the segment of chromosome 22 translocated to the partner chromosome in the cases associated with 22q translocation. The signals observed on the der(22) chromosome for LM confirm that the distal part of the der(22) is not translocated to another chromosome and strengthen the possibility of an interstitial deletion suggested previously by cytogenetic analysis for this cell line.

Microsatellite Analysis. Microsatellite markers were chosen in the proximal and telomeric parts to extend the previous LOH analysis (13) to both extremities of chromosome 22 long arm. The results are summarized in Fig. 2.

Interestingly, the seven cases containing two apparently normal chromosomes 22 showed LOH or homozygosity either at all loci tested (cell lines LP and G401) or at all loci except those in the centromeric part (cell lines AS, MT, KD, WT, and 2004). In the cell lines 2004 and KD, the different size of the two chromosome 22 short arms confirms the heterozygosity retention demonstrated in the proximal part of chromosome 22q. Together with the cytogenetic observations and the present FISH data, we can conclude that these seven cell lines have acquired a partial or putative complete isodisomy (Fig. 3). Definitive demonstration of complete isodisomy, although strongly suggested for G401 and LP, would require studying 22p polymorphic markers, which are not yet available.

For the cell line Wa2, the retention of heterozygosity was demonstrated along chromosome 22q except for seven microsatellite markers located in the RT critical region (13). Because only one signal has been obtained after *in situ* hybridization with the *hSNF5/INI1* probe, we can conclude that the *hSNF5/INI1* inactivation for Wa2 is due to the deletion of a limited part of one chromosome 22 including one *hSNF5/INI1* allele.

In the last four cases (DL, MON, TM87, and LM), all of which have karyotype changes involving chromosome 22q, the homozygous

Table 1 *Detailed karyotypes and FISH data in the RT cell lines*

Cell lines	Karyotypes	792F9		N96A6/77A2		Tel 22	
DL	46, XY, t(1;22)(p36;q11.2)	+	der(22)	−	−	+	der(1)
TM87	46, XY, t(11;22)(p15.1;q11.23)	+	der(22)	−	−	+	der(11)
LM[a]	46, XX, 3p+, 22q−	+	der(22)	−	−	+	der(22)
MON	46, XX, t(7;22)(q31;q11.2)	+	der(22)	−	−	+	der(7)
Wa2	46, XY, t(18;22)(p21;p11.2)	+	der(22)	−	der(22)	+	der(22)
G401	46, XY	+	+	−	−	+	+
LP	46, XY	+	+	+	+	+	+
WT	46, XX, −15, +der(15) t(1;15)(cen;cen)	+	+	+	+	+	+
MT	46, XY	+	+	+	+	+	+
AS	46, XX	+	+	+	+	+	+
2004[a]	46, XY	+	+	+	+	+	+
KD[a]	46, XX, 9p+	+	+	+	+	+	+

[a] 22p size polymorphisms allow discrimination of the two chromosomes 22.

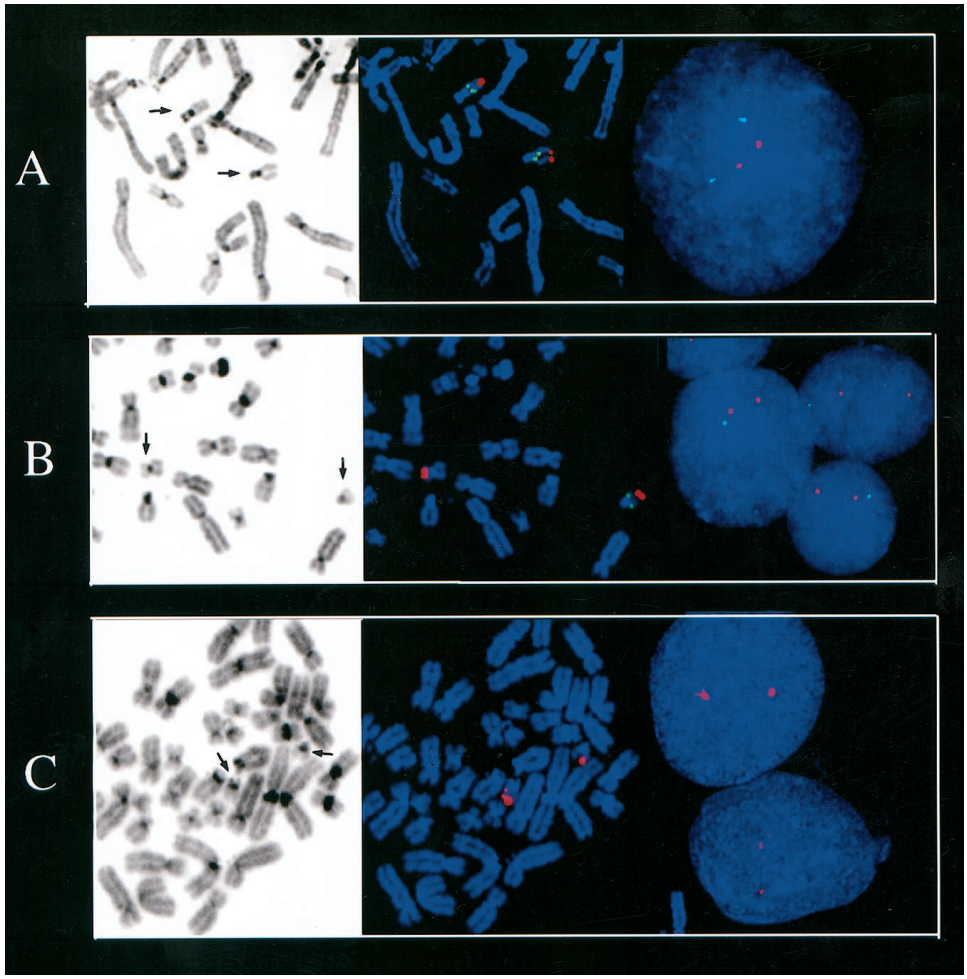

Fig. 1. Bicolor FISH performed on metaphase and interphasic nuclei. *A*, KD cell line; *B*, Wa2 cell line; *C*, LM cell line. Cosmid probes N96A6/77A2 are shown in *green*. The telomeric chromosome 22 probe is shown in *red*. *Black arrows* indicate both chromosomes 22 or der(22) in every cell line.

deletion of the consensus rhabdoid region was clearly demonstrated by either the absence of the corresponding PCR amplification or the absence of both *hSNF5/INI1* corresponding loci on the normal and the translocated chromosome 22. The retention of heterozygosity has been confirmed at both extremities of chromosome 22q for these four cases. The presence of a single allele at numerous contiguous polymorphic loci near to or in the proximity of the RT consensus region has been noted for DL, TM87, and LM, indicating that the size of the deletion was not identical on both chromosomes. Proximal inversion involving the *BCR* region and deletion in the distal part have been described previously for the der(22) in TM87 (10). In contrast, for MON, for which constitutional DNA was available, no loci demonstrated LOH, suggesting that the size of the homozygous deletion was similar on both alleles.

DISCUSSION

Our present study on the chromosomal mechanisms involved in the inactivation of the *hSNF5/INI1* gene in RTs combined the cumulated advantages of working simultaneously at both the cytogenetic and molecular levels on tumors that were perfectly well characterized for the gene mutation or deletion status (13).

The present FISH data and microsatellite analysis allow us to understand how the apparently normal karyotypes often described in

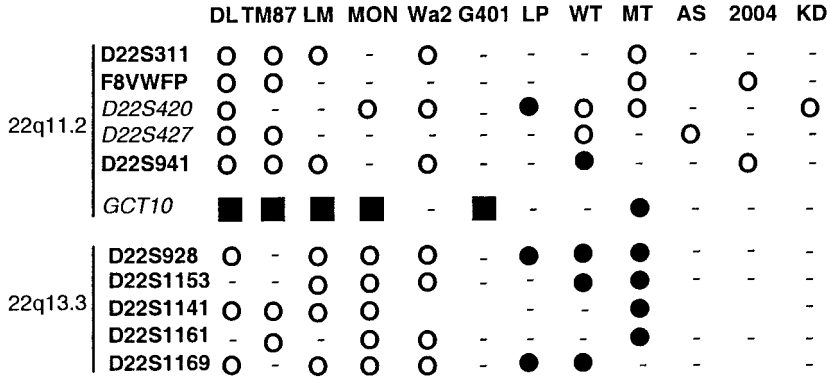

Fig. 2. Analysis of polymorphic markers tested on both extremities of chromosome 22q. *Italic*, those markers that have been described previously (13). ○ and ●, retention of heterozygosity and LOH of a marker sequence, respectively. *Dash*, the presence of a single PCR product in constitutional and/or tumor DNA. ■, homozygous deletions. Retention of heterozygosity is shown in both the proximal and distal part for the cell lines DL, TM87, LM, MON, and Wa2, whereas retention of heterozygosity is exclusively shown in the proximal part of 22q11.2 for cell lines WT, MT, AS, 2004, and KD. For G401, AS, 2004, and KD, homozygosity of 22q markers was detected at numerous loci (13); for LP and G401, a putative complete loss of chromosome 22 is suggested.

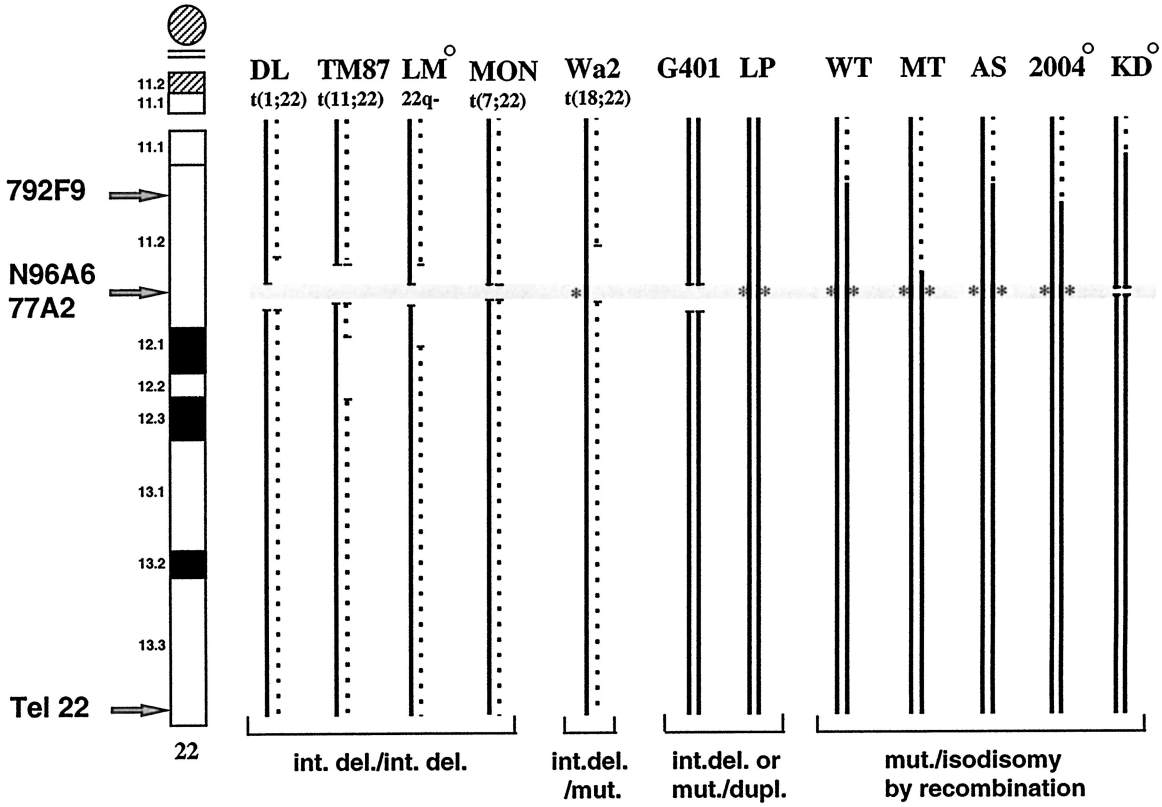

Fig. 3. Schematic representation of cytological and molecular data on the RT cell lines. FISH probes are approximately positioned by *arrows*. *Solid black line* and *dotted line*, the parental chromosomes. *Internal gap*, a deletion. ○, the presence of two different short arms of chromosome 22. ∗, point mutations (13) 196/dup17bp, 47TAC/TAA, 317/del1bp, 31/ins72bp, 37/del19bp, and 258/del13bp-ins2bp for Wa2, LP, WT, MT, AS, and 2004, respectively. Four groups are indicated according to the rearrangements involved: interstitial deletion and/or mutation associated or not associated with isodisomy resulting from duplication or recombination.

RTs hide the underlying complexity of the chromosome rearrangements involved (Fig. 3). On the whole, processes that lead to partial or complete isodisomy such as mitotic recombination occurring in the proximal part of chromosome 22q (demonstrated in five cases) or nondisjunction/duplication (highly suspected in two cases) appear to be major mechanisms associated with the *hSNF5/INI1* inactivation. Such isodisomy accompanies each of the present RTs exhibiting two cytogenetically normal chromosomes 22. It results in homozygosity for the mutation at the *hSNF5/INI1* locus. An alternate mechanism accounting for the *hSNF5/INI1* inactivation observed in our RT series is homozygous deletion in the rhabdoid consensus region. This was noticed in each of the four tumors carrying a chromosome 22q abnormality and, in particular, in the three tumors with chromosome translocations. This suggests that although they do not directly split the gene, these translocations may be involved in the recombination process leading to deletion. Only one case of our series illustrates the mutation/deletion classical model proposed for double-hit inactivation of a tumor suppressor gene.

The presence of low-copy number repeat families has been reported in proximal 22q (28, 29) in a region subject to several constitutional or somatic chromosome rearrangements. Constitutional chromosome changes have been reported in the t(11;22) of the general population and in the "cat eye" and the Di George syndromes. Translocations leading to different neoplasias such as the t(8;22) of Burkitt's lymphoma and the t(9;22) of chronic myeloid leukemia as well as the chromosome rearrangements in RT are included in the same region. Sequencing DNA at the deletion breakpoints would help to establish whether these low-copy number repeats predispose to recombination or deletion events.

Isodisomy has been documented in a number of cancer types using a combination of cytogenetic and molecular approaches. Indeed, isodisomy for chromosomes 13 and 11 has been observed in retinoblastoma and Wilms' tumors, respectively (15–19). Similarly, isodisomy for chromosome 3, which presumably encodes several tumor suppressor genes, is a frequent characteristic of non-small cell lung carcinoma, pancreatic adenocarcinoma, and uveal melanoma (30–32). However, the precise mechanisms leading to isodisomy have rarely been documented. Interestingly, both mitotic recombination and chromosome loss with reduplication occur spontaneously with similar frequencies at the HLA-A locus (33).

In RTs, we show that mitotic recombination leading to acquired homozygosity for most of chromosome 22 and for the *hSNF5/INI1* mutation occurs in close to one-half of the cases. This contrasts with the rarity of isodisomy for this same chromosome, which is frequently deleted in meningioma (34). Altogether, these data suggest that the chromosome mechanism underlying the inactivation of a tumor suppressor gene could be tissue or tumor specific.

RT diagnosis is often difficult. From now on, the involvement of *hSNF5/INI1* in RTs offers attractive new possibilities to help or to complete the clinicopathological approach to the disease. Although the presence of two *hSNF5/INI1* loci does not rule out the diagnosis of RT, our study indicates that analysis of the *hSNF5/INI1* locus by FISH can be of diagnostic interest in around one-half of RT cases by documenting hemizygous or homozygous deletion at this locus.

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