Molecular Analysis of the 9p21 Locus and p53 Genes in Ewing Family Tumors

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SUMMARY: The *EWS-ETS* rearrangements, and their respective fusion gene products, are specifically associated with histopathologically Ewing family tumors (EFT). These translocations are implicated in generating malignant transformation of EFT, but the presence of additional genetic alterations must be considered in the pathogenesis of such tumors. We analyzed 26 samples (biopsies and/or nude mice xenotransplants) collected from 19 patients with an EFT to determine whether molecular and cytogenetic alterations of the G₁/S checkpoint genes are implicated in the pathogenesis of EFT. We found inactivating p53 mutations in three (16%) cases, which correlated with a loss of p21^{WAF1/Cip1} expression and with a monosomy of chromosome 17 in two cases. Homozygous deletion of the p16^{INK4A}/p14^{ARF} gene was detected in four (21%) cases, three with codeletion of the p15^{INK4B} gene and with chromosome 9 abnormalities. In all of these cases, expression of the implicated genes was absent. Hypermethylation of the p16^{INK4A} and p15^{INK4B} genes was detected in two (10%) and three (16%) cases, respectively, and was correlated with a low level of gene expression. Neither cyclin D1, nor MDM2 and CDK4 amplification was observed. Kaplan-Meier analysis showed that patients with tumors carrying homozygous deletion of the 9p21 locus, or point mutations of the p53 gene, had poorer outcomes than those without these molecular alterations ($p = 0.005$). In conclusion, 58% (11 of 19) of the analyzed patients showed genetic or epigenetic alterations in either the 9p21 locus or p53 tumor suppressor genes, defining a subgroup of patients with poor clinical outcome. This fact points to an important role of the G₁/S cell cycle checkpoint dysregulation in the pathogenesis of EFT. (*Lab Invest* 2001, 81:803–814).

To progress through the cell cycle, mammalian cells must overcome the quiescent state of G₀ and enter the active G₁ cell cycle phase. In the G₁/S cell cycle checkpoint control, cells integrate mitogenic and antimitogenic stimuli to decide to undergo division (Johnson and Walker, 1999; Liggett and Sidransky, 1998). This checkpoint is regulated mainly by the cyclin-dependent kinases (CDK4 or CDK6) bound to the D-type cyclins. These complexes phosphorylate to the product of the Rb tumor suppressor gene (pRb) (Graña and Reddy, 1995; Sherr, 1996) and result in release of the E2F transcription factors that play a fundamental role in preparing the nucleus for DNA replication (Graña and Reddy, 1995; Huschtscha and Reddel, 1999; Johnson and Walker, 1999; Liggett and Sidransky, 1998; Sherr, 1996; Weinberg, 1995). CDK activity is influenced by a number of negative regulators: CDK inhibitors (CDKI) that physically associate with cyclins, CDK, or their complexes (Martín-Castellanos and Moreno, 1997). Among these CDKI are p16^{INK4A} (MTS1, CDKN2) (Kamb et al, 1994; Serrano et al, 1993) and p15^{INK4B} (MTS2) (Hannon and Beach, 1994), which form binary complexes exclu-

sively with CDK4 and CDK6, inhibiting their function and, by so doing, inhibiting pRb phosphorylation during G₁ (Graña and Reddy, 1995; Huschtscha and Reddel, 1999; Johnson and Walker, 1999; Liggett and Sidransky, 1998; Sherr, 1996; Weinberg, 1995). p16^{INK4A} and p15^{INK4B} genes map to the short arm of chromosome 9 (9p21–22), where they are found in tandem, spanning a region of approximately 80 kb, with p15^{INK4B} located approximately 25 kb centromeric to the p16^{INK4A} gene. The p15^{INK4B} gene is composed of two exons and its protein is a transforming growth factor- β (TGF- β)-induced inhibitor (Hannon and Beach, 1994). The p16^{INK4A} gene contains three exons and exhibits two distinct transcripts derived from two alternative first exons, exon 1 α and exon 1 β (13 kb centromeric to 1 α). The β transcript contains a 5' initiation codon in exon 1 β and incorporates the exon-2 coding sequence in a reading frame different from that of the α transcript (Stone et al, 1995). The p16^{INK4A} protein is specified by the α transcript (exons 1 α , 2, and 3), and the alternatively spliced β transcript (exons 1 β , 2, and 3) encodes the p14^{ARF} protein in humans (p19^{ARF} in mouse), which is a potent regulator of the cell cycle, functioning in a manner different from the CDKI (Liggett and Sidransky, 1998; Mao et al, 1995; Stone et al, 1995). p14^{ARF} acts upstream in the p53 pathway by binding to MDM2 and preventing p53 degradation, thus permitting p53-induced apoptosis or growth arrest (Lundberg and Weinberg, 1999; Sharpless and DePinho, 1999).

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The p53 protein, whose activity is modulated through its union to MDM2, also affects the G₁ to S progression by increasing expression of another CDKI, p21^{WAF1/Cip1}, which is able to inhibit any cyclin-CDK complexes, including D-type cyclin-CDK4/6 complexes (Johnson and Walker, 1999).

Molecular alterations in any of these genes affect the normal G₁ to S transition and play an important role in human tumorigenesis. In this sense, mutations affecting the p53 tumor suppressor gene are the most common genetic alterations yet identified in sporadic human tumors (Hollstein et al, 1999), followed by genetic alterations of the 9p21 genes that have been demonstrated in a wide variety of neoplasms. However, the overall incidence of alterations is higher in tumor-derived cell lines than in primary tumors (Spruck et al, 1994).

Homozygous deletion of the 9p21 locus occurs frequently in bladder carcinomas, gliomas, T-cell acute lymphoblastic leukemias, melanomas, and renal carcinomas (Drexler, 1998; Kamb et al, 1994; Liggett and Sidransky, 1998; Nobori et al, 1994). These genes are also commonly inactivated by methylation of the 5' CpG islands of their promoter region in head and neck, lung, brain, breast, colon, esophageal, and bladder cancer (Esteller et al, 2000; Gonzalez-Zulueta et al, 1995; Merlo et al, 1995). On the other hand, aberrant methylation status has been found exclusively at the p15^{INK4B} gene in a high proportion of gliomas and leukemias (Drexler, 1998). Point mutation is a less common mechanism of inactivation of the 9p21 locus genes (Huschtscha and Reddel, 1999).

Ewing family tumors (EFT) are a subset of pediatric bone tumors, which include Askin tumors, Ewing's sarcoma, and peripheral primitive neuroectodermal sarcoma (pPNET). EFT are specifically associated with cytogenetic rearrangements (Delattre et al, 1994), with the resultant fusion genes involving EWS gene and members of the ETS family of transcription factors, which from a clinical point of view provide a valuable diagnostic and prognostic marker (de Alava et al, 1998a, 1998b; Kovar, 1998; Llombart-Bosch, 1999; Llombart-Bosch et al, 1990, 2000; Patiño-García and Sierrasesúmag, 1997; Pellin et al, 1994; Turc-Carel et al, 1988). *EWS-ETS* rearrangements are implicated in generating malignant transformation of EFT, but the presence of additional genetic alterations must be considered in their pathogenesis (Kovar, 1998; Llombart-Bosch, 1999). These genetic alterations might not necessarily be tumor-specific, but might display interindividual variation that could account for differences in EFT phenotype, as well as in clinical behavior, differentiation capacity, invasive potential, and treatment resistance (Kovar, 1998; Llombart-Bosch, 1999). Hence, knowledge of the nature of additional aberrations in EFT might assist subclassification and provide prognostic tools. In this sense, among genes implicated in the G₁/S checkpoint, the p16^{INK4A} and p15^{INK4B} genes are lost from 0% to 30% in primary tumors (Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999; Park et al, 1999; Patiño-García and Sierrasesúmag, 1997; Wei et al, 2000), and 52%

in established cell lines (Kovar et al, 1997). The frequency of p53 mutations in primary tumors is less than 20% (de Alava et al, 2000; Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999; Patiño-García and Sierrasesúmag, 1997), as opposed to a mean frequency of 40% to 60% in most human malignancies (Hollstein et al, 1999). Neither pRb molecular alterations nor D1-type cyclin genetic amplification are observed in EFT (Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999), although occasional low-level amplification of MDM2 and CDK4 has been reported (Kovar, 1998; Ladanyi et al, 1995).

The present study was undertaken to better understand the frequency of the molecular alterations of those tumor suppressor genes located on chromosome 9p21–22 in 26 samples (primary tumors and/or nude mice xenotransplants) collected from 19 patients with an EFT. We also evaluated alterations of p53 and genetic amplification of the cyclin D1, MDM2, and CDK4 genes in the same samples and analyzed their association with 9p21 locus alterations and their cytogenetic repercussion to determine whether, and to what extent, these genes are implicated in the development and progression of this subset of pediatric bone tumors.

Results

Analysis of the 9p21 Locus Genes

Homozygous deletion of the 9p21 locus genes was not found in any primary tumor (PT), although deletion of p16^{INK4A} was observed in 4 of 12 nude mice xenotransplants (NMX), of which three also had codeletion of the p14^{ARF} and p15^{INK4B} genes. NMX from Case 19 maintained the exon 1β of p14^{ARF} and the exons 1 and 2 of p15^{INK4B} (Fig. 1; Table 1). In all of these samples, neither p16^{INK4A} nor p14^{ARF} and p15^{INK4B} transcripts were detected by RT-PCR, including in the NMX of Case 19. Thus, from all the patients analyzed, 4 of 19 (21%) presented homozygous deletion of the p16^{INK4A}/p14^{ARF} gene, from

Figure 1.

Representative analysis of the homozygous deletion of the 9p21 locus genes. Case 8 shows a complete deletion of these genes, whereas NMX from Case 19 retains the p15^{INK4B} gene and the exon 1β of the p14^{ARF} gene. MWM, molecular weight marker; PT, primary tumor; NMX, nude mice xenotransplant; WC, water control.

Table 1. Cytogenetic and Molecular Analysis of Individual Patients

Case	Specimen	Cytogenetics			EWS/ETS transcripts ^b	Deletion				Methylation			Amplification		Mutation	
		Specific translocation	Chr. 9	Chr. 17		p15-1	p16-1 α	p16-2	p14 ^{ARF} -1 β	p15	p16	MDM2	CD1	CDK4		
1	PT	t(11;22) ^a	Not known	Not known	—											135:TGC/TTC Cys→Phe
2	PT	t(11;22)	diploid	diploid	EWS 7/FLI-1 6											
3	NMX	Not known	Not known	Not known	EWS 7/FLI-1 6					A ^d						
4	PT	t(10;11;22)	+9 (18%) ^a	diploid	EWS 10/FLI-1 6											NA ^d
	NMX	t(10;11;22)	+9 (65%) ^a	diploid	EWS 10/FLI-1 6					A ^d						
	Recurrence	t(10;11;22)	+9 (43%) ^a	diploid	EWS 10/FLI-1 6					A ^d						
	Metastasis	t(10;11;22)	+9 (47%) ^a	diploid	EWS 10/FLI-1 6	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	NA ^d	
5	NMX from metastasis	t(10;11;22)	+9 (72%) ^a	diploid	EWS 10/FLI-1 6	A ^d	A ^d	A ^d	A ^d							NA ^d
	NMX	Not known	Not known	Not known	EWS 7/FLI-1 6											
6	PT	t(11;22)	t(9;?)(p22;?)	diploid	EWS 7/FLI-1 6	NA ^d	NA ^d	NA ^d	NA ^d							NA ^d
7	NMX	t(11;22) ^a	Not known	Not known	EWS 7/FLI-1 6	A ^d	A ^d	A ^d	A ^d							
8	PT	t(11;22)	diploid	diploid	EWS 7/FLI-1 5					A ^d						213: CGA/CGG Polymorphism
9	NMX	t(9;11;22;13)	t(9;11;22;13)	diploid	EWS 7/FLI-1 5	A ^d	A ^d	A ^d	A ^d							
9	PT	t(11;22)	diploid	diploid	EWS 7/FLI-1 6											138: GCC/GAC Ala → Asp
10	PT	Not known	Not known	Not known	EWS 7/FLI-1 6											
11	NMX	t(17;2;22;13)	diploid	translocated	EWS 7/FEV 2					A ^d						
12	PT	t(11;22)	diploid	diploid	EWS 7/FLI-1 7									A ^d		A ^d
13	NMX	t(11;22) ^a	Not known	Not known	EWS 7/FLI-1 7									A ^d		
13	NMX	t(11;22)	diploid	diploid	EWS 7/FLI-1 6											A ^d
14	NMX	t(11;22)	diploid	diploid	EWS 7/FLI-1 6									A ^d		
15	NMX	t(7;22)	diploid	diploid	EWS 7/FLI-1 6											151: CCC/CGC Pro → Arg
16	PT	t(11;22)	diploid	—17	EWS 7/FLI-1 6											
17	NMX	t(2;22)	diploid	diploid	EWS 7/FEV 2 ^c											A ^d
18	PT	Not known	Not known	Not known	EWS 7/FLI-1 6											
19	PT	t(11;22)	diploid	diploid	EWS 7/FLI-1 6											
	NMX	Not known	Not known	Not known	EWS 7/FLI-1 6					A ^d						

PT, primary tumor; NMX, nude mice xenotransplant.

^a Cytogenetic changes analyzed by FISH.^b Extra numbers in transcripts column refer to exons.^c Case with EWS/FEV transcripts previously reported (Lombart-Bosch et al. 2000).^d No data, normal; A, altered; NA, not available.

which three (16%) were accompanied by the p15^{INK4B} gene. Cytogenetically, two of these cases showed translocations involving chromosome 9p21–22 (Table 1). It is noteworthy that in Case 4, in particular, from which samples of PT, NMX from PT, recurrence, metastasis, and NMX from metastasis were available, deletion of the 9p21 locus genes (Fig. 2A), with the corresponding loss of expression (Fig. 2B), was always detected, except in the PT sample (Table 1). This indicates that this molecular alteration persisted during the tumoral progression and that it was already present in the PT. Although no abnormalities of chromosome 9 were detected in the metaphases from the direct samples of this case, by the fluorescence in situ hybridization (FISH) technique, a progressive gain of material of chromosome 9 was seen in the interphase nuclei from the successive samples of this particular case (Table 1).

Samples without homozygous deletion of the 9p21 genes were analyzed for the methylation status of the 5'CpG island of the p15^{INK4B} and p16^{INK4A} genes. Aberrant hypermethylation in the promoter region of the p16^{INK4A} gene was observed in Cases 12 (Fig. 3) and 14 (Table 1). For p15^{INK4B} methylation status, Cases 3, 7, and 10 were found to be hypermethylated (Fig. 3; Table 1). Hypermethylation of both genes in the same sample was not coincident, but was associated with a low level of expression of the corresponding gene.

p53 Analysis and p21^{WAF1/Cip1} Expression

We analyzed exons 5 through 8 of the p53 tumor suppressor gene because this region accounts for approximately 80% of the conserved domains of the gene and is the location of more than 95% of the mutations reported in sporadic tumors.

We detected point mutations in three samples corresponding to Cases 3, 11, and 16, making an incidence of 16% of the cases studied. All mutations were located in exon 5 of the p53 gene (Table 1), and are correlated—except for Case 16 where no RNA was available—with the low level or loss of p21^{WAF1/Cip1}

Figure 3.

Representative analysis of the methylation status of the p16^{INK4A} and p15^{INK4B} tumor suppressor genes. *M*, methylated; and *U*, unmethylated primers.

gene expression, ratio p21^{WAF1/Cip1}/β-actin = 0.0784 or 0 AU (arbitrary units) for Cases 3 and 11, respectively (Fig. 4). Nonrandom loss of one homolog of chromosome 17 was detected in Cases 11 and 16.

Recognition of the R213R polymorphism at exon 6 of the p53 gene was observed in Case 8 (Table 1).

Amplification of the MDM2, Cyclin D1, and CDK4 Genes

All cases were also analyzed for the MDM2, cyclin D1, and CDK4 amplifications by dPCR methods; however, no amplifications in any of these genes were observed.

Table 1 summarizes the results obtained from the analysis of the status of the 9p21 locus for p53, MDM2, cyclin D1, and CDK4 alterations. There was no association between p16^{INK4A}/p14^{ARF} status and p53 mutations. The sample from Case 3, however, showed p53 mutation with hypermethylation of the p15^{INK4B} gene. As a whole, 58% (11 of 19) of the analyzed patients showed genetic or epigenetic alterations in one or more of the p16^{INK4A}, p15^{INK4B}, p14^{ARF}, or p53 tumor suppressor genes, indicating the important role of these regulators of the G₁/S checkpoint in the pathogenesis of EFT.

Molecular Alterations and Follow-Up

The median follow-up for 18 of 19 patients was 22.5 months (range, 5–150 months), from which four re-

Figure 2.

Case 4. A, Deletion of the 9p21 genes was not found in the primary tumor (PT); however, it was observed in the nude mice xenotransplant (NMX) from the PT, in the recurrence (R) and in the NMX from metastasis (M). B, Deletion of the 9p21 genes was correlated with the loss of their expression. *WC*, water control; *rt-C*, RT-PCR control.

Figure 4.

Inactivating point mutation of the p53 tumor suppressor gene corresponding to Case 11, which correlates with the loss of p21^{WAF1/Cip1} gene expression.

maintained alive and without evidence of disease during this period (Table 2). Association between molecular findings and follow-up indicated that none of the surviving patients presented molecular alterations of the G₁/S checkpoint regulator genes. However, homozygous deletion of the 9p21 locus or point mutations of the p53 gene were present in tumors of

patients with the poorest outcomes (Fig. 5A; Table 3). Moreover, when complete inactivation of the 9p21 locus (homozygous deletion) or p53 genes was considered as a whole, as representative of the inactivation of the G₁/S checkpoint, we observed that patients with an EFT carrying any of these alterations showed an overall survival time shorter than those without these alterations (Fig. 5B; Table 3).

Discussion

Genetic mutations in the regulatory genes implicated in cell cycle control can be considered to target one of two critical pathways: the pRb pathway that regulates G₁/S-phase transition and the p53 pathway that induces growth arrest or apoptosis in response to either DNA damage or inappropriate mitogenic stimuli (Sharpless and DePinho, 1999). Molecular alterations of the integrating elements of either pRb, p53, or both pathways suppress their normal function, abrogating regulation of cell cycle transition from G₁ to S-phase and driving cells to proliferation. In EFT, these molecular alterations might constitute part of a multistep process with equivalent cellular effects, of which the *EWS-ETS* genetic fusion seems to be the initiating mechanism of EFT pathogenesis (Kovar, 1998; Llombart-Bosch, 1999).

Currently, in EFT, few studies have reported the frequency of homozygous deletions of the p16^{INK4A} tumor suppressor gene, which is normally accomplished with the codeletion of the p15^{INK4B} and p14^{ARF} genes, being of one-third for PT and more than 50% in tumor cell lines from unrelated patients (Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999; Park et al, 1999; Patiño-García and Sierrasesúmaga, 1997;

Table 2. Clinical Data for Patients

Case	Age/Sex	Primary site	Treatment	Follow-up	
				Metastasis	Last known status
1	20/M	Scapula	S+CT+RT	None	Alive and NED 48 months
2	2/M	Bone marrow	N/A	N/A	N/A
3	13/M	Pubis	S+CT+RT	Multiple	Death 5 months
4	11/F	Perone	S+CT+RT+T	Lung	Death 19 months
5	16/M	Soft tissue thigh	S+CT	None	Alive and NED 150 months
6	12/F	Homoplat	S+CT+RT	Multiple	Death 10 months
7	38/M	Retroperitoneum	S+CT+RT	Multiple	Death 22 months
8	14/M	Soft tissue thigh	S+CT+RT+T	Multiple	Death 9 months
9	20/M	Femur	S+CT+RT	Lung	Death 12 months
10	19/M	Rib	S+CT+RT	Lung	Death 47 months
11	24/M	Retroperitoneum	S+CT	Multiple	Death 12 months
12	9/M	Radius	S+CT	None	Alive and NED 138 months
13	21/F	Soft tissue thigh	S+CT+RT	Multiple	Death 48 months
14	16/M	Soft tissue ankle	S+CT+RT	Multiple	Death 78 months
15	20/F	Pelvis	S+CT+RT	Multiple	Death 14 months
16	16/M	Vertebral	S+CT+RT	Lung	Death 23 months
17	15/M	Soft tissue inguen	S+CT+RT+T	Multiple	Death 28 months
18	35/M	Soft tissue thigh	S+CT+RT	None	Alive and NED 90 months
19	17/M	Scapula	S+CT	Multiple	Death 19 months

S, surgery; CT, chemotherapy; RT, radiation therapy; T, autologous bone marrow transplantation; NED, no evidence of disease; N/A, information not available.

Figure 5.

Kaplan-Meier survival plots showing the prognosis for patients with (A) homozygous deletion of the 9p21 locus genes or (B) inactivation of G₁/S checkpoint (homozygous deletion of the 9p21 or inactivating point mutations of the p53 tumor suppressor gene).

Table 3. Kaplan-Meier Analysis of Molecular Alterations of the 9p21 Locus and p53 Tumor Suppressor Genes with Follow-Up

Molecular status	n	Median (months)	Range (months)	Log Rank	p value
Homozygous deletion of the 9p21 genes					
Absent	14	28	5–150	6.14	0.0132
Present	4	10	9–19		
Hypermethylation of p15 ^{INK4B} /p16 ^{INK4A}					
Absent	13	19	9–150	0.18	0.6689
Present	5	47	5–138		
Molecular alterations of the 9p21 genes ^a					
Absent	9	28	12–150	0.82	0.3646
Present	9	19	5–138		
Point mutations of p53					
Absent	15	28	9–150	3.62	0.0571
Present	3	12	5–23		
Homozygous deletion of the 9p21 genes or p53 point mutations					
Absent	11	47	10–150	7.88	0.0050
Present	7	12	5–23		
Molecular alterations of the 9p21 genes or p53 point mutations					
Absent	7	48	12–150	1.88	0.1704
Present	11	18	5–138		

^a Molecular alterations of the 9p21 genes include epigenetic and genetic changes (hypermethylation and homozygous deletion).

Tsuchiya et al, 2000; Wei et al, 2000). These percentages were clearly higher than those observed in other soft-tissue sarcomas in which incidence of molecular alterations of the 9p21 locus genes ranged from 0% to 15% (Meye et al, 1998; Orlow et al, 1999; Schneider-Stock et al, 1997; Wei et al, 1999; Yao et al, 1998), indicating that inactivation of these tumor suppressor genes might play an important role in the pathogenesis of EFT. On the other hand, p53 mutations have been reported in approximately 20% of EFT (de Alava et al, 2000; Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999; Patiño-García and Sierrasesúmaga, 1997), whereas neither molecular alterations of the pRb gene nor D1-type cyclin genetic amplification have been observed (Kovar, 1998; Kovar et al, 1997; Llombart-Bosch, 1999), although an occasional low

level of MDM2 and CDK4 amplification has been reported (Kovar, 1998; Ladanyi et al, 1995). However, amplification of the 12q13 locus, where MDM2 and CDK4 are located, is a frequent event in other soft-tissue sarcomas (Khatib et al, 1993; Wei et al, 1999).

Among the main mechanisms of the 9p21 locus gene inactivation are deletion and hypermethylation of the 5'CpG islands of their promoter region (Esteller et al, 2000; Gonzalez-Zulueta et al, 1995; Herman et al, 1996; Kamb et al, 1994; Merlo et al, 1995). In this sense, in our study we identified deletion of the p16^{INK4A}/p14^{ARF} gene in four (21%) cases, of which three had also codeletion of the p15^{INK4B} gene and aberration of chromosome 9. The NMJ from Case 19 maintained exon 1β of the p14^{ARF} gene and the two exons of the p15^{INK4B}. Nonetheless, no expression of

these genes was observed. A cryptic microdeletion, not observed by cytogenetics, affecting the complete sequence of the p16^{INK4A} gene, including exon 2 shared with p14^{ARF}, might be the basis for this finding, as well as in the loss of the p15^{INK4B} expression. A similar case with a deletion adjacent to p15^{INK4B}, affecting the p16^{INK4A}/p14^{ARF} gene, was previously reported (Jen et al, 1994).

On the other hand, methylation analysis, by methylation-specific PCR (MSP), showed p16^{INK4A} hypermethylation in 10% of the patients, whereas p15^{INK4B} hypermethylation was observed in 16%. In our cases, this alteration never correlated with the total loss of RNA expression because of the incomplete hypermethylation of the p16^{INK4A} and p15^{INK4B} alleles, as the amplification with unmethylated primers showed. In Case 12, such PT as its NMJ presented showed hypermethylation of the p16^{INK4A} gene, suggesting that some tumor cells, with inactivated p16^{INK4A} gene in the PT, provided some proliferative advantage that was maintained through their growth in the NMJ. Contrary to deletion, p16^{INK4A} and p15^{INK4B} genes never are cohypermethylated in the same sample, indicating that this epigenetic change is a more selective mechanism of gene inactivation whose role in EFT tumorigenesis remains to be clarified.

In Cases 4, 6, and 19, with paired samples of PT and NMJ from PT, we observed deletion of the 9p21 genes in the NMJ but not in the PT. The study of surgical specimens from many human cancer categories reveals a lower proportion of cases with 9p21 deletion than cell lines established from them (Cairns et al, 1994; Drexler, 1998; Kamb et al, 1994; Kovar et al, 1997; Nobori et al, 1994; Okamoto et al, 1994), because admixed nonneoplastic cells render the demonstration of deletion by differential PCR more difficult, and/or because small clones of tumoral cells within the tumor carrying 9p21 deletion might not be detected. Many cancers contain cell populations that escape from the normal limitations on proliferative potential, becoming immortals (Huschtscha and Reddel, 1999). A frequent change observed in these immortalized cells is the loss of p16^{INK4A} function (Huschtscha and Reddel, 1999; Okamoto et al, 1994). Thus, it might be easier to establish cell lines from tumors that have undergone molecular alterations of the 9p21 locus during their development in vivo (Drexler, 1998; Huschtscha and Reddel, 1999), although these alterations were present in only a small proportion of cells. These observations are similar to our cases with respect to the NMJ. Available samples from Case 4 especially reinforce this theory. Molecular alterations of the 9p21 locus were not found in the PT, although in the NMJ from the PT, recurrence and, in the NMJ from the metastasis, loss of expression and homozygous deletion of these genes were observed. From the metastasis we were able to confirm only the loss of expression. These results show that deletion of the 9p21 locus was clearly manifested during tumor progression and that it was already present in the primary sample, but in a small tumoral cell population. We reached this conclusion because homozygous

deletion of 9p21 was detected in the NMJ from the PT and in the recurrence and metastasis. Direct association between the detection of 9p21 deletion by differential PCR with the gain of chromosome 9, observed by FISH, suggests that cells with an extra copy of chromosome 9 are carriers of a cryptic microdeletion of the 9p21 locus. In fact, we were not able to detect this deletion in the PT where the percentage of cells with a gain of chromosome 9 was only 18% compared with the rest of the samples with a higher proportion of cells with gains of chromosome 9. Thus, loss of 9p21 locus in this case confers a proliferating advantage that selects this tumoral clone.

We found a significant association with a poor overall survival in those patients with an EFT carrying homozygous deletion of the 9p21 locus. Kovar et al (1997) were the first to suggest the prognostic implication of the 9p21 alterations in patients with Ewing sarcoma. Later, Tsuchiya et al (2000) and, more clearly, Wei et al (2000) confirmed this. The study of Wei et al is particularly important because it is the only one that, in a multivariate analysis of disease-specific survival, found 9p21 locus deletion to be a significant negative prognostic factor, independent of stage at diagnosis. Unlike the study of Wei et al, we also analyzed the effect of the methylation status of the p16^{INK4A}/p15^{INK4B} genes on overall survival. Our data suggest that 9p21 homozygous deletion, but not p16^{INK4A}/p15^{INK4B} hypermethylation, adversely affects the prognosis of EFT patient.

We also detected inactivating mutations of the p53 gene in three (16%) cases, which correlated with low or no expression of the p21^{WAF1/Cip1} gene, and with nonrandom loss of one homolog of chromosome 17 in two cases. EFT patients with point mutations of p53 showed a trend toward a poorer overall survival, which agrees with studies reported by Abudu et al (1999) and de Alava et al (2000), based on immunohistochemical analysis, which show that p53 alterations appear to define a clinical subset of patients with a markedly poor outcome that is independent of site, local treatment, or necrosis of the tumors. Thus, in spite of the rarity of abnormalities of the p53 gene in EFT, they might indicate the assumption of a more aggressive outcome constituting an independent poor prognostic factor in EFT (Abudu et al, 1999).

The recent evidence that the p16^{INK4A}/p14^{ARF} locus encodes two products (Sharpless and DePinho, 1999) that affect the pRb and p53 pathways, positions the p16^{INK4A}/p14^{ARF} gene at the nexus of the two most critical tumor-suppressor pathways governing neoplasia. Homozygous deletion of the 9p21 locus deregulates the G₁/S-phase transition by causing the simultaneous loss of p16^{INK4A} activity and p53 activity, because of the absence of p14^{ARF} (Sharpless and DePinho, 1999). Nonetheless we did not find any significant differences for the p21^{WAF1/Cip1} expression between those cases with and without alterations of the 9p21 locus, indicating that inactivation of the p16^{INK4A}/p14^{ARF} gene might not be functionally equivalent to alterations in p53, but still might produce aggressive biologic behavior. In our cases, p53 point

mutations were not associated with molecular alterations of the 9p21 locus; however, as a whole, they define a subset of patients whose tumors present the G₁/S checkpoint clearly disrupted, and whose clinical evolution was the poorest. The possible significance of the inactivated G₁/S checkpoint in identifying a subset of EFT patients with a poor prognosis merits a more systematic combined analysis of the 9p21 locus and p53 alterations to stratify patients into favorable and unfavorable groups, who might benefit from alternative treatments.

We did not detect genetic alterations of the CDK4 and MDM2 genes, both located in the chromosome 12q13, a region frequently amplified in non-EFT soft-tissue sarcomas (Khatib et al, 1993; Wei et al, 1999). In our study, in all cases cytogenetically analyzed, chromosome 12 was apparently normal. However, deletion of the 9p21 eliminates p16^{INK4A} and p14^{ARF} genes, which interact with the CDK4 and MDM2, respectively. Hence, alterations of the 9p21 and 12q13 might be redundant, and although they functionally represent similar effects, they are clearly implicated in the development of different pathologies.

In conclusion, our study shows that the genetic and epigenetic alteration in the 9p21 locus eliminates three important cell-growth inhibitors (p16^{INK4A}, p14^{ARF}, and p15^{INK4B}) but constitutes the second molecular event in importance, after the EWS/ETS rearrangements, in the pathogenesis of EFT. Alterations in the p53 gene have been observed to a lesser extent. However, as a whole, 58% of the analyzed patients showed alterations of these important G₁/S checkpoint regulators. In this sense, the Kaplan-Meier analysis showed that patients with EFT carrying homozygous deletion of the 9p21 locus or p53 point mutations (ie, with inactivated G₁/S checkpoint control) had a significantly poorer outcome than those without evidence of these molecular alterations. This fact points to an important role for the G₁/S cell cycle checkpoint dysregulation in the pathogenesis and clinical behavior of EFT, indicating that further studies with more patients and with combined analysis of 9p21 homozygous deletions and p53 abnormalities are necessary.

Material and Methods

Samples and Patients

We analyzed freshly frozen material from 26 samples, including PT and/or NMJ, from 19 patients with an EFT admitted to the Department of Pathology at the Medical School in Valencia, Spain. Some cases had been characterized previously by ultrastructural and immunohistochemistry (Llombart-Bosch et al, 1990). The most relevant clinical data from our cases are shown in Table 2. Follow-up was available in 18 patients (median, 22.5 months; range, 5–150 months); four patients survived without evidence of disease for this period. Presence of EWS/ETS rearrangements was confirmed by RT-PCR in all tumor samples, except for Cases 1 and 15 for which no RNA was available, but with the t(11;22) and t(7;22) confirmed

by FISH and cytogenetics respectively (Table 1). Samples from PT, NMJ from PT, recurrence, metastasis, and NMJ from metastasis were available from Case 4 (Table 2).

DNA and RNA were isolated from frozen tissue samples. High molecular weight DNA was obtained with proteinase K (Life Technologies, Gaithersburg, Maryland) digestion and purification by a conventional phenol-chloroform protocol, whereas total RNA extraction was performed according to the manufacturer's instructions using TRIzol Reagent (Life Technologies) 1 ml/50 mg of tissue. Both DNA and RNA were quantified by spectrophotometry at 260 nm, and RNA integrity was checked by 1% ultraPURE agarose (Life Technologies, Paisley, Scotland) gel electrophoresis and ethidium-bromide staining.

Analysis of the 9p21 Locus

Homozygous Deletion of the p15^{INK4B}, p16^{INK4A}, and p14^{ARF} Genes. Differential PCR (dPCR) was used to detect homozygous deletion of the 9p21 genes. In this assay, a simultaneous amplification of genomic DNA was performed by using two sets of primers, one to the target gene sequence under study and the other to target the β -globin gene, which was used as internal control for DNA quality and loading. Each 25- μ l PCR reaction contained 250 ng of genomic DNA, 1x PCR buffer II (100 mM Tris-HCl, 500 mM KCl, pH 8.3) (Perkin-Elmer, Norwalk, Connecticut), 2 mM MgCl₂, 0.2 mM dNTPs (Life Technologies), 10% DMSO (Sigma Chemical, St. Louis, Missouri), 0.6 U AmpliTaq (Perkin-Elmer) and 0.2 μ M for each set of primers (PE-Applied Biosystems, Warrington, United Kingdom). PCR was carried out in a DNA Thermal Cycler 9600 (Perkin-Elmer) wherein the samples were preheated at 95° C for 5 minutes and amplified as follows: 30 cycles for p15^{INK4B} exon 1 and p16^{INK4A} exon 2, 35 cycles for p16^{INK4A} exon 1 α , and 28 cycles for p14^{ARF} exon 1 β , with a final extension step of 72° C for 10 minutes. Primers, annealing temperature, and PCR product sizes are summarized in Table 4. The amplification products were visualized on an ethidium-bromide-stained 1.5% agarose gel. The presence of homozygous deletion was determined by the loss of the PCR product corresponding to p15^{INK4B}, p16^{INK4A}, and p14^{ARF} genes with the presence of the β -globin amplification. A water control, with no DNA, was included in each experiment.

Methylation-Specific PCR (MSP). The methylation status of the 5'CpG island in the promoter region of the p15^{INK4B} and p16^{INK4A} genes was determined following the procedure described by Herman et al (1996). Chemical DNA modification was performed with the CpGenome DNA modification kit (Oncor, Gaithersburg, Maryland) from 1 μ g of DNA. The purified DNA was eluted into 35 μ l of water. A 2- to 4- μ l aliquot of template was amplified by PCR using specific primers for the methylated or unmethylated sequences (Table 4). After 40 cycles of amplification in the presence of 1x PCR buffer II, 2 mM MgCl₂, 0.2 mM dNTPs, 0.15 μ M of unmethylated or methylated prim-

Table 4. Primers Used for dPCR, RT-PCR, and MSP

Determination	Primer sequence (5' → 3')		Size, bp	Annealing temp, °C
Gene deletion	Sense	Antisense		
p15 exon 1	CCAGAAGCAATCCAGGCGCG	AATGCACACCTCGCCAACG	570	60
p16 exon 1 α	GAAAGGAGAGGAGGGGCT	GCGCTACCTGATTCCAATTC	350	50
p16 exon 2	GGAATTTGAAACTGGAAGC	TCTGAGCTTTGGAAGCTCT	525	55
p14 ^{ARF} exon 1 β	TCCCAGTCTGCAGTTAAGG	GTCTAAGTCGTTGTAACCCG	439	55
β -globin	GAAGAGCCAAGGACAGGTAC	CAACTTCATCCACGTTCCAC	268	55–60
RT-PCR				
p15	CCAGAAGCAATCCAGGCGCG	CGTTGGCAGCCTTCATCG	725	60
p16	GGCCCTGTAGGACCTTCG	GGAGCAGCATGGAGCCG	588	60
p14 ^{ARF}	CCCTCGTGCTGATGCTACTGA	ACCAACCAGCGTGTCCAGGAA	202	57
p21 ^{Cip1}	CTCAGAGGAGGCGCCATG	GGGCGGATTAGGGCTTCC	521	57
β -actin	GAGCGGGAAATCGTCCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG	234	60
MSP ^a				
p15M	GCGTTGCTATTTTGGGTTT	CGTACAATAACCGAACGACCGA	148	60
p15U	IGIGATGIGTTTGTATTTGTGGTT	CCATACAAAACCAACCAACCAA	154	60
p16M	TTATTAGAGGGTGGGGCGGATCGC	CCACCTAAATCGACCTCCGACCG	234	65
p16U	TTATTAGAGGGTGGGGTGGATTGT	CCACCTAAATCAACCTCCACCAA	234	60
Gene amplification				
MDM2	GAGGGCTTTGATGTTCTCTGA	GCTACTAGAAGTTGATGGC	143	53
DR	CCACTGAATCTGCTCCTGGTATG	GTGTGGCATAGTAGTTGTAGTGG	114	53
CDK4	CATGTAGACCAGGACCTAAGG	GGAGGTCGGTACCAGAGTG	539	57
β -globin	GAAGAGCCAAGGACAGGTAC	CAACTTCATCCACGTTCCAC	268	57
Cyclin D1	TTCTGCCCTTTGATGTTAC	AGGCTGAATCAATGTCTT	115	50
INF- γ	TCTTTTCTTTCCCGATAGGT	CTGGGATGCTCTTCGACCTC	150	50
p53 analysis				
Exon 5	TTCCTCTTCTGCAGTAC	GCCCCAGCTGCTCACCA	214	55
Exon 6	GGGCTGGTTGCCAGGGT	TAGTTGCAAACCAGACCTC	150	60
Exon 7	GTGTTGTCTCTTAGGTTG	TGGCAAGTGGCTCCTGAC	142	55
Exon 8	CCTATCCTGAGTAGTGGT	GTCTGCTTGCTTACC	166	55

dPCR, differential PCR; RT-PCR, reverse transcription PCR; MSP, methylation-specific PCR.

^a Sequence differences between modified primers and unmodified DNA are in boldface type, and differences between methylated/modified and unmethylated/modified are underlined.

ers, and 1 U of AmpliTaq Gold (Perkin Elmer), the PCR products were analyzed on a 2% ethidium-bromide-stained agarose gel. The presence of methylation was confirmed by the identification of the PCR bands in those samples amplified with the methylated primers. All of the cases tested were evaluated for the presence of an unmethylated-specific fragment, which served as internal control for the quality of the treated DNA.

Expression of the p15^{INK4B}, p16^{INK4A}, and p14^{ARF} Genes. Reverse transcription was performed with a GeneAmp RNA-PCR kit (Perkin-Elmer) under the following conditions: aliquots of 2 μ g of total RNA were incubated with 1 μ g of 50 μ M random hexamers. After incubation at 70° C for 10 minutes, 4 μ l of 25 mM MgCl₂, 2 μ l of 10 mM dNTPs, 200 U of MuLV reverse transcriptase, 20 U of RNase inhibitor, and 2 μ l 10-fold synthesis PCR buffer II were added to complete the 20 μ l of reverse transcription mixture. After 10 minutes at room temperature, the mixture was incubated at 42° C for 30 minutes, followed by 94° C for 5 minutes to stop reverse transcription, and 5° C for 5 minutes. PCR amplification was performed in duplicate in a 25- μ l reaction mixture containing 2 μ l of cDNA product as a template, 1.5 to 2.5 mM MgCl₂, 0.2 mM dNTP, 1 $\times$ PCR

buffer II, 5% to 10% DMSO, 1.5 U AmpliTaq Gold, and 0.15 μ M of each set of primers (Table 4). cDNA preparations were also amplified in duplicate in a parallel reaction with β -actin primers (Table 4). Cycling programs for PCR (40 cycles for p15^{INK4B} and p16^{INK4A}, 30 cycles for p14^{ARF}, and 25 cycles for β -actin) were optimized to ensure the lineal amplification and to make a semiquantitative estimation of the generated PCR products comparing band intensity from p15^{INK4B}, p16^{INK4A}, and p14^{ARF} with those generated with the β -actin primers after image analysis (1D Image Analysis Software, Eastman Kodak, Rochester, New York). PCR products corresponding to each case were electrophoresed in the same 1.5% ethidium-bromide-stained agarose gel, visualized, photographed, and analyzed.

p53 Mutation Analysis and p21^{WAF1/Cip1} Expression

p53 Sequencing Analysis. High molecular weight DNA was used in four different PCR-amplification reactions (exons 5 to 8) performed in 50 μ l of reaction mixture containing approximately 100 ng of genomic DNA, 1 $\times$ PCR buffer II, 1.5 mM of MgCl₂, 0.5 μ M for

each primer (Table 4), 0.2 mM of dNTP, and 1 U of AmpliTaq. After 35 cycles, PCR products were purified with Centricon columns (Amicon, Beverly, Massachusetts) and sequenced on an Applied Biosystem model 310 automated sequencer (Foster City, California) using the dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems; Warrington, United Kingdom).

RT-PCR of p21^{WAF1/Cip1}. Analysis of p21WAF1/Cip1 RNA expression was performed as described above for the p15^{INK4B}, p16^{INK4A}, and p14^{ARF} genes, but with 25 cycles of PCR amplification and a 57° C annealing temperature (Table 4).

Analysis of the MDM2, CDK4, and Cyclin D1 Gene Amplification

Gene amplification of the MDM2, CDK4, and cyclin D1 genes was determined by dPCR using two sets of primers, one corresponding to the target gene and the other to an internal control gene sequence; the dopamine receptor (DR), the β -globin, and the interferon gamma (INF γ) genes for MDM2, CDK4, and cyclin D1 analysis, respectively (Table 4). PCR-reactions were performed in 25 μ l of a solution containing 250 ng of DNA, 1 $\times$ PCR buffer II, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.6 AmpliTaq, and 0.2 μ M for each primer. Annealing temperatures are indicated in Table 4. After 28 cycles of amplification, PCR products were visualized on a 1.5% to 2.5% ethidium-bromide-stained agarose gel. A higher band intensity for the target genes than the reference bands confirmed a gene amplification. Positive, negative, and water controls were employed in each experiment.

Cytogenetic Studies and FISH Technique

Tumor cells from the original specimens, as well as from xenografted tumors, were harvested after 24 hours (direct preparations) and after 3 to 7 days in culture. The specimens were processed as previously described (Llombart-Bosch et al, 1990; Noguera et al, 2001).

FISH was performed on tumor cells for chromosomes and nuclei after short-term culture using standard procedures. Analysis of t(11;22) was performed using two combinations of probes: chromosome 11 α -satellite centromere-specific probe (D11Z1) and coatasome 22 total chromosome probe (wcp22) and coatasomes 11 and 22 total chromosome probes (wcp11/wcp22). Coatasome 9 total chromosome probe (wcp9) was used in Case 4 (Noguera et al, 2001). All probes were obtained from Oncor.

Statistical Analysis

The Kaplan-Meier proportional risk test (log rank) was used to study the effect of the molecular alterations analyzed on overall survival. This test is included in the SPSS statistical package (version 9.0).

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