

Ewing Family Tumors: Potential Prognostic Value of Reverse-Transcriptase Polymerase Chain Reaction Detection of Minimal Residual Disease in Peripheral Blood Samples

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ABSTRACT

In more than 95% of patients, the Ewing family of tumors (ET) has chimeric transcripts caused by fusion of the EWS gene to either FLI1 or ERG. The presence of specific EWS-FLI1 or EWS-ERG transcripts in peripheral blood (PB) samples of patients being treated for ET was prospectively evaluated, and these data were correlated to their clinical status. The authors studied 113 PB samples from 28 patients with ET. Treatment included chemotherapy, radiotherapy, and surgical excision of tumor after induction therapy. PB samples were taken prospectively at least 2 weeks after resection of tumor. Nested reverse-transcriptase polymerase chain reaction (RT-PCR) followed by Southern blot was performed in all samples. Resected tumors were reviewed for the degree of response to chemotherapy and volume. Seventy-seven PB samples from 28 patients had EWS-FLI1/ERG transcripts. In 11 patients, PB samples became negative with treatment, and, in 5 of them, the samples remained negative throughout the study. Samples taken during progression were always positive and, in 4 patients, became positive before progression was clinically evident. All patients with transcripts other than EWS-FLI1 type 1 (n = 3) died from tumor progression. This is a sensitive assay to monitor circulating tumor cells in Ewing tumors. The preliminary data suggest that progression is preceded by positive samples and may be related to specific transcript types.

KEY WORDS

Ewing's sarcoma; Primitive peripheral neuroectodermal tumor; Minimal residual disease; Reverse-transcriptase polymerase chain reaction; Southern blot; Circulating tumor cells.

INTRODUCTION

The Ewing sarcoma family of tumors (ET) is defined by the presence of a gene fusion involving the EWS gene and a member of the ETS family of transcription factors, usually FLI1 or ERG (5). This entity includes Ewing's sarcoma/primitive peripheral neuroectodermal tumor of bone and soft tissues, and several special clinicopathologic presentations (i.e. Askin tumor). Stage at diagnosis remains the main prognostic factor in ET for disease-free and overall survival (23). Metastases at diagnosis, which are usually detected by imaging techniques, occur in approximately 15% to 25% of patients (9,23) and are associated to a poor prognosis. Neoadjuvant therapy, combining systemic control by chemotherapy with local control by surgery and radiotherapy, has dramatically improved survival rates. Moreover, initial response to therapy, usually assessed histologically on surgical specimens as the degree of tumor viability (19,23,26), has become another major independent prognostic factor, especially in localized tumors of the extremities.

Gene fusions in ET are transcribed into chimeric transcripts, which can be detected using reverse-transcriptase polymerase chain reaction (RT-PCR) in over 95% of patients. Their detection is helpful in the diagnosis of ET (7,13,22). It has been recently reported that patients with EWS-FLI1 type 1 transcripts have a better prognosis than all other patients, regardless of other prognostic factors (2).

The high sensitivity of PCR allows the detection of as little as 1 tumor cell in 10^6 normal cells (7). Consequently, several groups have reported detection of tumor cells in peripheral blood (PB) and bone marrow (BM) samples of patients with ET (17,18,24,27). Those studies could be potentially useful to assess the initial response to treatment and the presence of minimal residual disease during and after treatment.

Contradictory results have been reported regarding the value of RT-PCR performed on BM samples of patients with ET at the time of diagnosis to predict relapses (8,29). However, BM involvement in ET has been reported to be probably multifocal (15). Therefore, in contrast to previous approaches, we studied serial PB samples during and after therapy and correlated the presence of circulating tumor cells with other prognostic factors, including stage at diagnosis, histologic response to therapy, transcript type, and clinical outcome.

MATERIALS AND METHODS

Twenty-eight patients with ET (14 males and 14 females, mean age 12.3 y, range, 3 to 18 y), were included in this prospective study. Using standard criteria, 22 tumors were diagnosed as Ewing's sarcomas, and 6 were diagnosed as primitive peripheral neuroectodermal tumors (24). Five patients (patients 1 to 5) had metastatic disease at diagnosis; in the other 23 patients (patients 6 to 28), tumors were localized. Fifteen tumors were located in the extremities and 13 in the central axis. All patients were diagnosed and treated at Clínica Universitaria de Navarra, Pamplona, Spain. Multimodal therapy included chemotherapy (52 weeks, 18 cycles based on VAC [vincristine, actinomycin, and cyclophosphamide] plus ifosfamide), radiotherapy, and surgical excision (Fig. 1). Induction of treatment included 12 weeks of chemotherapy, 5

weeks of radiotherapy, and surgical resection of tumor 5 weeks after radiotherapy. Chemotherapy continued after induction up to 18 cycles (approximately 40 more weeks).

This study included 113 PB samples. PB samples always were taken at admission of the patient immediately before administration of a chemotherapy cycle. Because blood contamination by ET cells released during surgical procedures has been reported (30), we began to collect samples after induction and at least 2 weeks after tumor resection. Four ml of EDTA-treated whole blood were centrifuged on a Ficoll gradient (25 minutes, 1000 g). The mononuclear cell layer was used for RNA extraction. In addition, samples of the primary tumors were available for molecular study in all patients. Informed consent was obtained from the patients or their guardians, and the Local Ethics Committee approved the protocol.

Surgical resection specimens were available for histologic study in 20 patients. Two independent observers evaluated tumor necrosis on the surgical specimens. A whole section of the tumor was studied, including an average of 8 blocks. The tumors were graded according to the following system (19): grade 1, a tumor in which in gross examination at least one nodule of viable tumor is seen; grade 2, a tumor with only isolated microscopic foci of viable tumor cells; and grade 3, no viable tumor can be found. Tumor volume was calculated based either on the imaging studies (computed tomography and magnetic resonance imaging) or on the tumor resection specimens. Volume calculation formulas for elliptical (bony) and spheroidal (soft tissue) tumors are described in detail elsewhere (19).

Molecular Study

Extraction of total RNA was based on a modified guanidinium isothiocyanate-phenol chloroform method using the TRIZOL (Gibco BRL) reagent. An assay was designed including a nested RT-PCR using appropriate primers for EWS and FLI1/ERG genes. Primers have been described elsewhere (3,18) and are listed in Table 1. The assay was performed using 1 µg of total RNA as template and a GeneAmp RNA-PCR Kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ, U.S.A.) following the manufacturer's recommendations. Reverse transcription included an incubation period of 45 min at 42°C with random hexamers followed by a 5-min period at 99°C to denature the enzyme. MgCl₂ concentration in the RT was 5 mM. PCR was then performed with an initial denaturation step of 95°C for 2 min, followed by 35 cycles including a denaturation step at 94°C for 30 sec, annealing at 66°C (68°C for the second round) for 30 sec, and extension at 72°C for 60 sec, with 7 min of extension after the completion of the last cycle. A second round of amplification was performed using one-fifth of the initial PCR product with a second set of specific internal primers (see Table 1). Products were first analyzed by a 1% agarose gel electrophoresis and ethidium bromide staining. MgCl₂ concentration in both PCRs was 1.25 mM. All PCR products were then blotted onto nylon membranes (Hybond+; Amersham Pharmacia Biotech, Amersham, U.K.), and detected with internal probes (see Table 1) labeled with chemoluminescence (3' oligolabelling system; Amersham Pharmacia Biotech). EWS-FLI1 type 1 (EWS exon 7 joined in frame with exon 6 of FLI1), type 2 (EWS exon 7 joined in frame with exon 5 of FLI1), and EWS-ERG were typed according to the size of the RT-PCR products on the agarose gels and Southern blots. EWS-FLI1 7-7 products (EWS exon 7 joined in

frame with exon 7 of FLI1) were sequenced (21). Negative controls lacking either tumor RNA or reverse transcriptase were routinely used.

Statistical Study

Statistical tests included Pearson chi-square test and Fisher exact test for qualitative parameters and one-way ANOVA to compare qualitative parameters and tumor volume. They were performed using the SPSS statistical package, version 6.0 for Windows (SPSS, Chicago, IL, U.S.A.).

RESULTS

Molecular Results

Primary tumors

Primary tumors showed EWS-FLI1 type 1 fusions in 25 patients (Table 2). One patient (#5) had EWS-FLI1 type 2 transcripts. Patient 22 had an EWS-FLI1 fusion in which EWS exon 7 was joined in frame with exon 7 of FLI1, and patient 2 had EWS-ERG transcripts.

Peripheral blood samples

PB samples, when positive, showed the same transcript type as primary tumors. No changes in fusion transcript type were observed in any patient throughout the study. All patients had at least one positive PB sample (Table 2, Fig. 2). Seventy-seven samples had EWS-FLI1/ ERG transcripts (Table 2). Thirty-six of 113 PB samples studied (32%) did not show any chimeric transcript; all of these PB samples corresponded to patients with EWSFUJ type 1 transcripts in the primary tumor.

Clinical Outcome

Median follow-up time as of March 1, 1998 was 26 months (mean, 35 months; range 5 to 110). At that time, 20 of 28 patients (15 of 23 with localized disease and all patients with metastatic disease) had finished the entire treatment protocol (12 months); the remaining 8 patients were still being treated. Three patients of the 23 with localized disease and 4 of 5 with metastatic disease showed progression. Four patients died from disease: patient 1 at 14 months after diagnosis, patient 2 at 18 months, patient 5 at 57 months, and patient 22 at 42 months.

Molecular Results and Clinical Parameters

Molecular results are displayed in Figure 2 in relationship with stage and status of disease. In 5 patients whose initial sample was positive, subsequent samples were negative (Fig. 3). In another 6 patients, positive and negative results alternated. In patients with tumor progression, all samples taken during progression were positive (Fig. 4). Four patients had positive samples before progression was clinically evident;

the time elapsed between positive samples and progression was 2, 14, 34, and 36 months (Fig. 2). All three patients with transcripts other-than EWS-FLI1 type 1 (patients 2, 5, and 22) showed clinical progression and died from disease; patients 2 and 5 had metastatic disease at diagnosis and patient 22 with a 7-7 fusion had localized disease at diagnosis. Transcript type other than EWS-FLI1 type 1 was more frequently associated with progression ($p = 0.01$, Pearson chi-square with continuity correction) (Table 3).

Forty-six of 82 samples (56%) taken from patients with clinical remission were positive; in patient 28, two positive samples were observed 8 y after the end of therapy. However, positive samples were more prevalent in patients with progression (28 of 32; 88%) ($p = 0.04$, Pearson chi-square with continuity correction). Positive samples were more frequently found among patients with metastatic disease at diagnosis (21 of 23; 91%) than in patients with localized disease (56 of 90; 62%) ($p = 0.01$, Pearson chi-square with continuity correction).

Histologic Response to Therapy, Tumor Size, and Molecular Results

Complete histologic study could be performed in 20 patients for whom resected tumors were available. Eight tumors had grade 3 response, 10 had grade 2 response, and 2 had grade 1 response. The first PB sample after tumor resection (14 weeks) was positive in all patients with grade 1 and 2 responses, and in 5 of 6 patients with grade 3 response. Moreover, the degree of response to chemotherapy was not associated to any given pattern of molecular results (i.e. positive samples that became negative, alternating results, and samples that remained negative) (results not shown).

Mean tumor volume was 98 ml (range, 6 to 550 ml). Tumor volumes of patients with metastasis at diagnosis were higher than those with localized disease (276 versus 57 ml; $p = 0.04$). Tumor volume in patients in whom samples became negative with treatment was not significantly different from that of patients in whom samples remained positive (152 versus 40 ml, respectively; $p = 0.43$).

DISCUSSION

The role of pathologists in the management of ET is the accurate diagnosis and assessment of tumor necrosis after initial treatment (19,25). Detection of specific chimeric transcripts, already a useful routine assay in molecular pathology for the diagnosis of small cell developmental tumors (3,7,13,22), makes ET a good model to study minimal residual or metastatic disease (4,6,8,17, 18,24,27).

ET-specific fusion transcripts can become undetectable by RT-PCR assay during therapy, as occurred in 5 patients in this series. In one of these patients, samples remained negative during a follow-up of 2 y. All negative PB samples corresponded to patients that had complete remission at that time, a finding of clinical and biological value.

Transcript detection occurred in all patients with disease progression. Moreover, four patients had positive samples several months before progression was clinically evident.

Similar results have been already reported in several types of leukemia (20), suggesting that patients with repeated positive samples have a high probability for disease progression. These findings may have clinical implications, and therapy can be intensified in ET patients at high risk for disease progression. Ongoing quantitative RT-PCR assays probably will help to further assess the prognostic value of transcript detection.

Several patients maintained positive results after the end of therapy. Moreover, transcripts were still detectable in a few patients with a good clinical response to therapy. This is remarkable because most of them are considered to be in complete remission, and one of these patients has been off therapy for 8 y. Several authors reported sustained positive RT-PCR results (AML-ETO transcripts) even 8 y after induction of therapy in a few patients with acute myeloid leukemia (14). The consistent presence of transcripts in PB samples of patients in complete remission may represent tumor cells under the control of the immune system (1), or may indicate that another molecular event is needed to become metastatic. The high sensitivity of nested RT-PCR followed with Southern blot could allow detection of an amount of cells hypothetically too small to cause a relapse. Moreover, disappearance of tumor cells may not be a prerequisite for cure, as shown in a study using a quantitative assay in patients with childhood acute lymphoblastic leukemia (20). Because RNA is unstable and short-lived in the extracellular environment, the presence of RT-PCR positivity probably indicates intact tumor cells. Alternating results were seen in several patients, possibly because the number of circulating tumor cells may be close to the detection threshold of the RT-PCR assay, as described in acute lymphoblastic leukemia (16).

Tumor cells could be detected in our series after tumor resection regardless of stage at diagnosis, although they were detected more frequently in patients with metastatic disease at diagnosis, as previously reported (6,27). These data confirm that ET is a disseminated disease at diagnosis. Of note, the first samples taken after resection of tumor were frequently positive, even in patients with complete tumor necrosis, suggesting that tumor cells remain present in PB even in patients with complete responses to induction therapy. This represents further biological evidence supporting the need for systemic therapy after resection of the tumor. Detection of ET transcripts was independent of tumor volume, indicating that transcript detection in PB samples may not be a function of initial tumor burden, in contrast to the suggestions of several authors (27).

Fusion transcripts in ET show a considerable molecular heterogeneity at the level of exonic structure and in the involved ETS genes (31). We and others have recently shown that patients with ET with EWS-FLI1 transcripts other than type 1 have a worse prognosis (2,28) regardless of stage, tumor location, or age (2). However, the biological basis for this phenomenon is unclear and could include secondary molecular alterations in genes regulating cell cycle (12), drug resistance, or ability to metastasize. In the present study, patients with transcripts other than EWS-FLI1 type 1 repeatedly had positive samples, tumor progression, and death from disease in a short time. Moreover, all negative samples (36 of 113; 32%) were taken from patients with EWS-FLI1 type 1 fusions in the primary tumor and in initial PB samples. This is consistent with our previous results (2), and suggests the clinical usefulness of transcript detection in PB samples of patients with ET. Although the number of patients in our series with transcripts other than EWS-FLI1 type 1 is small, our results suggest that presence of

circulating tumor cells may not only depend on the tumor burden (quantitative factors), but also on the specific transcript variants (qualitative factors).

Whether PB samples or BM specimens are the best source to detect minimal residual disease in ET is a matter of discussion. One report describes BM as the most sensitive source (6). Nevertheless, BM involvement in ET is probably multifocal, and the amount of minimal residual disease can vary in BM samples obtained from different sites (10,11,15). From a practical point of view, this approach requires extensive BM investigation with several samples taken every few months. Moreover, disagreement exists to whether BM involvement at diagnosis measured by RT-PCR has prognostic value (8,29). Study of PB samples is probably more convenient for patients with ET because they are usually subjected to monitoring of blood counts during and after completion of therapy. A potential limitation of PB samples was reported by Zoubek et al. (30), who showed detection of ET cells in PB samples during open biopsies, but not before and after 6 days after surgery. Based on their results, we think that detection of ET cells 2 weeks after resection in our study was not caused by tumor cell mobilization.

Circulating tumor cells can be detected in PB samples of patients with ET. Although further studies with longer follow-up, BM samples, and quantitative techniques are warranted to assess the clinical value of this assay, our initial data suggest that it might be a useful additional approach to monitor therapy and may help to predict disease progression.

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REFERENCES

1. Bradstock KF, Gottlieb DJ. Interaction of acute leukemia cells with the bone marrow microenvironment: implications for control of minimal residual disease. *Leuk Lymphoma* 1995;18:1-16.
2. de Alava E, Kawai A, Healey JA, et al. EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. *J Clin Oncol* 1998;16:1248-55.
3. de Alava E, Ladanyi M, Rosai J, Gerald WL. Detection of chimeric transcripts in desmoplastic small round cell tumor and related developmental tumors by reverse transcriptase polymerase chain reaction. A specific diagnostic assay. *Am J Pathol* 1995;147:1584-91.
4. Costa J. Caution neophiles!. Editorial. *Lab Invest* 1997;77:211-2.
5. Delattre O, Zucman J, Melot T, et al. The Ewing family of tumors. A subgroup of small round cell tumors defined by specific chimeric transcripts. *N Engl J Med* 1994;331:294-9.

6. Dockhorn-Dworniczak B, Zoubek A, Kovar H, Craft A, Haas RJ, Gardner H, Jurgens H. Detection of Ewing's tumor cells in bone marrow and peripheral blood samples by means of RT-PCR. A preliminary report of the EICESS 92 Molecular genetic committee [abstract]. *Med Pediatr Oncol* 1996;27:242A.
7. Downing JR, Head DR, Parham DM, et al. Detection of the (11;22)(q24;q12) translocation of Ewing's sarcoma and peripheral neuroectodermal tumor by reverse transcription polymerase chain reaction. *Am J Pathol* 1993;143:1294-300.
8. Fagnou C, Michon J, Peter M, et al. Presence of tumor cells in bone marrow but not in blood is associated with adverse prognosis in patients with Ewing's tumor. *J Clin Oncol* 1998;16:1707-11.
9. Horowitz ME, Malawer MM, Woo SY, Hicks MJ. Ewing's sarcoma family of tumors: Ewing's sarcoma of bone and soft tissue and the peripheral primitive neuroectodermal tumor. In: Pizzo PA, Poplack DG, eds. *Pediatric oncology*, 3rd ed. Philadelphia: Lippincott-Raven. 1997:831-64.
10. Ito Y, Wasserman R, Galili N, Reichard BA, Shane S, Lange B, Royera G. Molecular residual disease status at the end of chemotherapy fails to predict subsequent relapse in children with B-lineage acute lymphoblastic leukemia. *J Clin Oncol* 1993; 11:546-53.
11. Kelly KM, Womer RB, Barr FG. Minimal disease detection in patients with alveolar rhabdomyosarcoma using a reverse transcriptase-polymerase chain reaction method. *Cancer* 1996;78: 1320-7.
12. Kovar H, Jug G, Aryee DNT, et al. Among genes involved in the RB dependent cell cycle regulatory cascade, the p16 tumor suppressor gene is frequently lost in the Ewing family of tumors. *Oncogene* 1997;15:2225-32.
13. Ladanyi M. The emerging molecular genetics of sarcoma translocations. *Diagn Mol Pathol* 1995;4:162-73.
14. Nucifora G, Larson RA, Rowley JD. Persistence of the 8; 21 translocation in patients with acute myeloid leukemia type M2 in long term remission. *Blood* 1993;82:712-5.
15. Oberlin O, Bayle C, Hartmann O, Terrier-Lacombe MJ, Lemerle J. Incidence of bone marrow involvement in Ewing's sarcoma: value of extensive investigation of the bone marrow. *Med Pediatr Oncol* 1995;24:343-6.
16. Ouspenskaia MV, Johnston DA, Roberts M, Estrov Z, Zipf T. Accurate quantitation of residual B-precursor acute lymphoblastic leukemia by limiting dilution and a PCR-based detection system: a description of the method and the principles involved. *Leukemia* 1995;9:321-8.
17. Peter M, Magdalenat H, Michon J, et al. Sensitive detection of occult Ewing's cells by the reverse transcriptase polymerase chain reaction. *Br J Cancer* 1995;72:96-100.
18. Pfliderer C, Zoubek A, Gruber B, et al. Detection of tumor cells in peripheral blood and bone marrow from Ewing tumor patients by RT-PCR. *Int J Cancer* 1995;64:135-9.
19. Picci P, Bohling T, Bacci G, et al. Chemotherapy-induced tumor necrosis as a prognostic factor in localized Ewing's sarcoma of the extremities. *J Clin Oncol* 1997;15:1553-9.
20. Roberts WM, Estrov Z, Ouspenskaia MV, Johnston DA, McClain KL, Zipf TF. Measurement of residual leukemia during remission in childhood acute lymphoblastic leukemia. *N Engl J Med* 1997; 336:317-23.

21. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463-7.
22. Sorensen PH, Liu XF, Delattre O, Rowland JM, Biggs CA, Thomas G, Triche Ti. Reverse transcriptase PCR amplification of EWS/FLI-1 fusion transcripts as a diagnostic test for peripheral primitive neuroectodermal tumors of childhood. *Diagn Mol Pathol* 1993;2: 147-57.
23. Terrier P, Llombart-Bosch A, Contesso G. Small round blue cell tumors in bone: prognostic factors correlated to Ewing's sarcoma and neuroectodermal tumors. *Semin Diagn Pathol* 1996;13:250-7.
24. Toretsky JA, Neckers L, Wexler LH. Detection of (11;22) (q24;q12) translocation-bearing cells in peripheral blood progenitor cells of patients with Ewing's sarcoma family of tumors. *J Natl Cancer Inst* 1995;87:385-6
25. Triche TJ. Pathology of pediatric malignancies. In: Pizzo PA, Poplack DG, eds. *Pediatric oncology*, 3rd ed. Philadelphia: Lippincott-Raven, 1997:141-86.
26. Valérdiz S, Pardo-Mindán FJ, Sierrasesúmaga L. Clinicopathological features of Ewing's sarcoma after radiochemotherapy. *Diagn Oncol* 1992;2:193-6.
27. West DC, Grier HE, Swallow MM, Demetri GD, Granowetter L, Sklar J. Detection of circulating tumor cells in patients with Ewing's sarcoma and peripheral primitive neuroectodermal tumor. *J Clin Oncol* 1997;15:583-8.
28. Zoubek A, Dockhom-Dwomiczak B, Delattre O, et al. Does expression of different EWS chimaeric transcripts define clinically distinct risk groups of Ewing tumor patients? *J Clin Oncol* 1996; 14:1245-51.
29. Zoubek A, Ladenstein R, Windhager R, et al. Predictive potential of testing for bone marrow involvement in Ewing tumor patients by RT-PCR: a preliminary evaluation. *Int J Cancer* 1998;79:5660.
30. Zoubek A, Kovar H, Kronberger M, Amann G, Windhager R, Gruber B, Gardner H. Mobilization of tumor cells during biopsy in an infant with Ewing sarcoma. *Eur J Pediatr* 1996;155:373-6.
31. Zucman I, Melot T, Desmarte C, et al. Combinatorial generation of variable fusion proteins in the Ewing family of tumors. *EMBO J* 1993;12:4481-7.

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Table 1. Sequences of primers and probes used in the molecular assays		
First PCR	EWS 22.3	5-TCCTACAGCCAAGCTCCAAGTC-3'
	FLI1 11.3	5-ACTCCCGTTGGTCCCCTCC-3'
	ERG 3	5'-ACTCCCGTTGGTGCCTTCC-3'
Nested PCR	EWS 22.4	5'-CCAACAGAGCAGCAGCTAC-3'
	FLI1 11.4	5'-CAGGTGATACAGCTGGCG-3'
	ERG 4	5'-CAGGTGATGCAGCTGGAG-3'
Probes	EWS 22.6	5'-CAGAGCAGCAGCTACGGGCAGC-3'
	FLI1 11.6	5'-TGCCACAGCTGGATCTG-3'

Table 2. Chimeric transcripts found in primary tumors and in PB samples		
Transcript type	Primary tumor	PB samples
EWS-FLI1 type 1	25	61
EWS-FLI1 type 2	1	9
EWS-FLI1 fusion 7-7	1	6
EWS-ERG	1	1
Negative	0	36*
Total	28	113
* All negative samples correspond to patients that had EWSFLI1 type 1 fusions in primary tumors.		

Table 3. Patients with tumor progression, and transcript type			
Transcript type	No progression	Progression	Total
EWS-FLI1 type 1	21	4	25
All other types	0	3	3
Total	21	7	28
p = 0.01, Pearson's chi-square with continuity correction.			

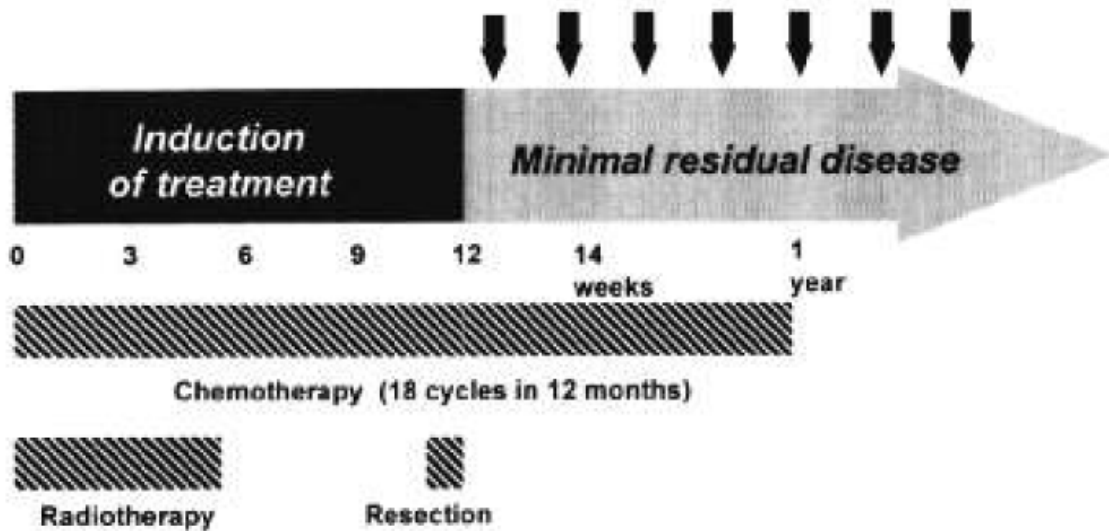


Figure 1. Treatment protocol and sample schedule. Arrows indicate sample timing.

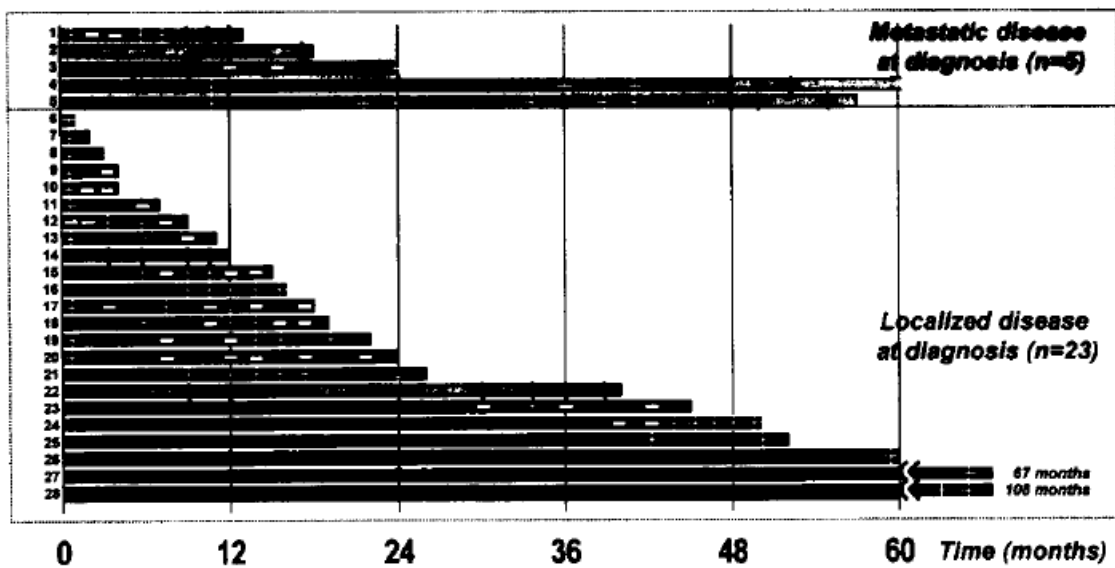


Figure 2. Molecular results with respect to stage and clinical status. Follow-up of patients is represented in a bar histogram in which the horizontal axis represents time. Filled segments of the bars represent complete remission, while open segments correspond to tumor progression. The first 12 months represent treatment.

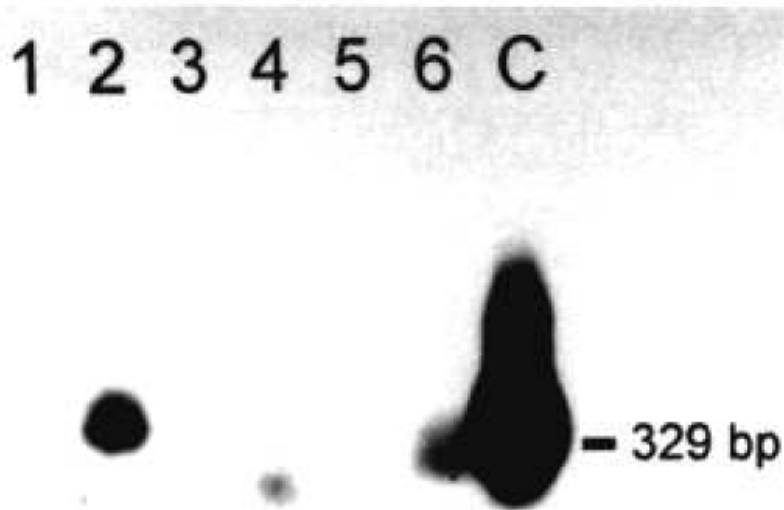


Figure 3. Southern blots of patients on treatment. Patient in lane 2 had a sample with EWS-FLI1 type 1 transcripts 4 weeks after tumor resection that became negative (lane 3) 2 months later (patient 20 in Fig. 2). Sample in lane 4 corresponds to patient 22 10 months from diagnosis. Sample in lane 5 was taken from patient 13 6 months after diagnosis. Lane 6 corresponds to a patient in whom samples remained positive during treatment (patient 21 in Fig. 2). 329 bp is the expected size for a EWS-FLI1 type 1 transcript. C, positive control; lane 1, negative control. Blot has been hybridized with FLI1 11.6 probe.



Figure 4. Southern blots of patients after treatment. Samples of patients in remission are analyzed in lanes 2 to 4. Patient in lane 4 (patient 21) shows a faint band corresponding to a EWS-FLI1 type 1 transcript. Patients in lanes 5 and 6 (patients 1 and 5 in Fig. 2, respectively) showed progression of disease. Patient in lane 6 shows a 395-bp band corresponding to a EWS-FLI1 type 2 fusion. Additional faint bands in lanes 5 and C presumably represent PCR artifacts. The expected size for a EWS-FLI1 type 1 transcript is 329 bp. C, positive control; lane 1, negative control. Blot has been hybridized with FLI1 11.6 probe.

