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Short Communication

Detection of the (11;22)(q24;q12) Translocation of Ewing's Sarcoma and Peripheral Neuroectodermal Tumor by Reverse Transcription Polymerase Chain Reaction

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Ewing's sarcoma and the related primitive neuroectodermal tumor (PNET) share a unique and specific t(11;22)(q24;q12) chromosomal translocation. The breakpoints have recently been cloned and shown to involve the* EWS *gene on chromosome 22 and the* FLI-1 *gene on chromosome 11. Translocation results in the fusion of these genes on the der(22) chromosome, resulting in the production of a novel chimeric* EWS/FLI-1 *message. Using oligonucleotide primers derived from* EWS *and* FLI-1 *complementary DNAs, we were able to amplify a specific fusion transcript from 18 of 18 cases containing t(11;22) and 10 of 14 cases of Ewing's sarcoma/PNET that had unsuccessful cytogenetics. No* EWS/FLI-1 *fusion transcripts were detected in five cell lines derived from cases of pediatric sarcomas having a histological diagnosis other than Ewing's sarcoma/PNET. The sensitivity and specificity of this PCR analysis demonstrates the usefulness of this approach for the primary diagnosis of t(11;22)-containing Ewing's sarcoma/PNET and for the detection of metastatic or residual disease. (Am J Pathol 1993, 143:1294–1300)

The differential diagnosis of small, round cell tumors of childhood often requires the use of the entire armamentarium of ancillary techniques at the disposal of the surgical pathologist. Ewing's sarcoma and the related primitive neuroectodermal tumor (PNET) are clear examples where electron microscopy, immunohistochemistry, and cytogenetics have aided not only in the accurate diagnosis of these tumors, but also in the demonstration of their histogenetic relationship to one another.[1,2] Both Ewing's sarcoma and PNET share a specific reciprocal translocation between band q24 on chromosome 11 and band q12 on chromosome 22.[3–7] In an analysis of 85 cases of Ewing's sarcoma and PNET, 88% of the cases were found to have either a standard t(11;22) or a complex translocation involving a third chromosome in addition to chromosomes 11 and 22.[4] Unfortunately, successful karyotypes in Ewing's sarcoma are obtained in less than half of the cases.[7–8]

The chromosomal breakpoints involved in t(11;22) have recently been identified at a molecular level and

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shown to involve the *EWS* gene on chromosome 22 and the *FLI-1* on chromosome 11.[9–10] The critical product resulting from this translocation seems to be the fusion of these genes on the der(22) chromosome, producing a novel chimeric *EWS/FLI-1* gene.[9] Supporting this interpretation is the observation that the *EWS/FLI-1* fusion transcript is readily identified in Northern blot analysis of cases containing a der(22) t(11;22), whereas expression of a reciprocal product from der(11) has not been observed.[9] Moreover, secondary loss of the der(11) chromosome has been reported in several cases of Ewing's sarcoma.[5,11]

The *EWS* gene encodes a protein of 656 amino acids of unknown function. Analysis of the primary sequence suggests that the EWS protein contains an amino terminal domain composed of a degenerate motif containing the dipeptide serine-tyrosine, which is repeated 31 times, and a central 85-amino acid region with homology to the RNA-binding domain of several proteins.[9] In contrast, *FLI-1* is the human homologue of the murine *Fli-1* gene that was first identified as a frequent site of retroviral integration in Friend murine leukemia virus-induced erythroleukemia.[12,13] *FLI-1* is a member of the *ets* proto-oncogene family of transcriptional factors.[12] The t(11;22) results in the deletion of the putative RNA-binding domain of *EWS*, and its replacement with the *ets*-like DNA binding domain of *FLI-1*.[9] Full characterization of the function of the *EWS/FLI-1*-encoded fusion product will be required before an understanding can be reached on the role this product plays in the transformation process.

The molecular characterization of the t(11;22) has provided the necessary information for the establishment of fluorescence *in situ* hybridization approaches for the detection of this translocation and for the design of oligonucleotide primers to amplify the fusion transcript by reverse transcription polymerase chain reaction (RT-PCR).[9,14,15] In this study, we describe the use of a sensitive and specific RT-PCR assay to determine the frequency of this tumor-specific translocation and to characterize the structure of the *EWS/FLI-1* fusion transcript in 32 cases of Ewing's sarcoma and PNET.

Materials and Methods

Patient Samples and Cell Lines

RT-PCR analysis for the presence of the *EWS/FLI-1* fusion transcript was performed on six established human Ewing's sarcoma cell lines and on clinical tumor preparations from an additional 26 patients having a primary diagnosis of either Ewing's sarcoma or PNET. Primary clinical samples were obtained from the Pediatric Oncology Group (16 patients) or St. Jude Children's Research Hospital (10 patients) and were stored as frozen cell suspensions before analysis. Wright-stained cytocentrifuge preparations of tumor cell suspension were analyzed for the percentage of tumor cells. Cytogenetics was performed using standard techniques and was successful on all of the cell lines and in 12 of 26 primary clinical samples.[5] All samples were obtained following informed parental consent. As negative controls for the RT-PCR analysis, five cell lines established from pediatric solid tumors having a diagnosis other than Ewing's sarcoma were used. These cell lines included, three rhabdomyosarcomas (SJ-RH1–3) and two neuroblastomas (SJ-NB1 and SJ-NB2).[16]

PCR Analysis

Total RNA was extracted and complementary DNA synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase as previously described.[17] For PCR amplification of the complementary DNA products, previously described primers (5′A and 3′A, Figure 1, B and C) were used at 0.5 μmol/L in 40 cycles of PCR amplification.[9] Addition of the co-solvent dimethylsulfoxide at a final concentration of 5% resulted in a 10-fold increase in the sensitivity of detection of the fusion product and was used in all experiments.[18] Nested primer PCR analysis was performed using 5% of the original PCR products and the oligonucleotide primers, 5′B and 3′B (Figure 1, B and C). Strict precautions against contamination were undertaken, and negative controls were included.[19] PCR products were electrophoresed through a 1.2% agarose gel, transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) and hybridized with a *FLI-1* specific oligonucleotide probe (Figure 1) end-labeled with ^{32}P. Autoradiography was performed using XAR-5 film (Kodak, Rochester, NY).

DNA Sequence Analysis

Sequencing was performed by the chain termination reaction modified for fluorescent-based DNA sequencing using an Applied Biosystems 373 DNA sequencer (Applied Biosystems Inc., New York, NY). PCR products cloned into the vector, pCRII (In-Vitrogen, San Diego, CA), were used as templates for sequencing.

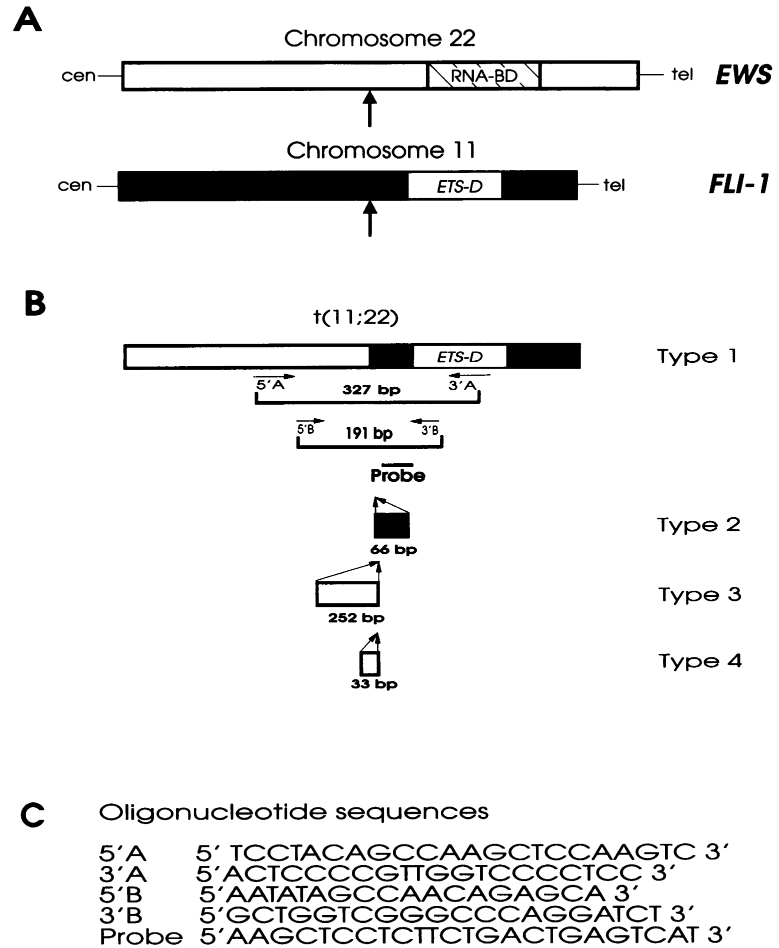


Figure 1. A: *Schematic diagram of the EWS and FLI-1 genes and their predicted protein products. The cross-hatched region in EWS corresponds to a domain with homology to RNA-binding proteins (RNA-BD), whereas the unshaded domain in FLI-1 corresponds to an ets-like DNA-binding domain (ETS-D).*[9] *The orientation of these genes in relationship to the centromere (cen) and telomere (tel) are indicated. The arrows indicate the relative position of the breakpoints resulting in a type 1 fusion product.* B: *Diagram of the t(11;22)-specific EWS/FLI-1 fusion transcripts (types 1 to 4) and the location of the oligonucleotide primers and probe used for the RT-PCR analysis. The size of the PCR products generated corresponds to those for a type 1 fusion transcript.* C: *Sequence of the PCR amplification and detection oligonucleotides.*

Results

Total RNA from six Ewing's sarcoma cell lines and 26 primary clinical samples diagnosed as either Ewing's sarcoma or PNET were subjected to RT-PCR analysis using *EWS* and *FLI-1* oligonucleotides. All of the cell lines and 12 of the primary clinical samples contained t(11;22), whereas the remainder had unsuccessful cytogenetics (Table 1). An initial RT-PCR reaction was performed using the 5′A and 3′A set of primers that bracket the translocation and are localized in the 28th repeat of the amino-terminal domain of *EWS* and the DNA binding domain of *FLI-1*, respectively (Figure 1, B and C).[9] To increase the sensitivity, a second round of amplification was performed on 10% of the positive cases and on all cases negative in the first round of

Table 1. *Clinical and RT-PCR Data on the 32 Cases of Ewing's/PNET*

		Type of fusion products				
	No	1	2	3	4	Neg.*
Cell lines						
Ewing's sarcoma	6	0	5	0	1	0
Other pediatric solid tumors	5	0	0	0	0	5
t(11;22)-containing cases						
Ewing's sarcoma	8	3	5	0	0	0
PNET	4	2	2	0	0	0
Cases without successful karyotypes						
Ewing's sarcoma	12	3	2	0	3	4
PNET	2	0	2	0	0	0

* Negative for the *EWS/FLI-1* fusion product.

amplification using the 5′B and 3′B oligonucleotides as nested primers (Figure 1, B and C).

As indicated in Table 1, all t(11;22)-containing cell lines and primary clinical samples were positive by RT-PCR analysis for the *EWS/FLI-1* fusion transcript. A representative autoradiogram from Southern analysis of the PCR products is shown in Figure 2, A and B. The level of expression of the *EWS/FLI-1* fusion transcript as detected by PCR analysis varied by up to fivefold among cases using the probe illustrated in Figure 1. Similar results were obtained using junction-specific probes to the previously described type 1 and type 2 *EWS/FLI-1* fusion transcripts (see below). This variability in the level of expression was not due to differences in the ability of the RNA to be amplified, as similar levels of the ubiquitously expressed *c-ABL* message were detected in each case (Figure 2). Accurate comparison of the levels of *EWS/FLI-1* expression to the percentage of tumor cells in the clinical samples could not be made; however, variability in the level of expression was also detected among the cell lines. No fusion transcript was detected in five pediatric sarcoma cell lines established from primary tumors having a diagnosis other the Ewing's sarcoma/PNET (Table 1).

When RT-PCR analysis was performed on 14 cases of Ewing's sarcoma or PNET that had unsuccessful cytogenetics, all but four cases expressed the *EWS/FLI-1* message (Table 1 and Figure 2C). The negative cases included three Ewing's sarcomas and one PNET. The diagnoses in these cases were based primarily on light microscopy with immunocytochemistry (HBA-71 positivity) and electron microscopy supporting this diagnosis in two cases.[1,20] Repeat analysis of these negative cases by both nested primer analysis with the indicated oligonucleotides (Figure 1) and by amplification with a third set of oligonucleotide primers hybridizing to regions 5′ and 3′ to the A-set of primers failed to reveal any expression of the *EWS/FLI-1* fusion transcript (negative data not shown). Moreover, in these cases, a sampling error seems unlikely because cytocentrifuge preparations of the material used for RNA extraction revealed high percentages of tumor cells. In addition, analysis of the sensitivity of this RT-PCR analysis demonstrated the ability to detect the *EWS/FLI-1* fusion transcript in 1 μg of total RNA containing as little as 50 to 100 pg of t(11;22)-containing RNA and from one tumor cell in 10^4 to 10^5 cells by a direct mixing experiment (data not shown). Taken together, these data suggest that the majority of Ewing's sarcoma and PNET express a *EWS/FLI-1* fusion transcript as a result of a t(11;22) translocation. However, a small percentage of cases having a histological diagnosis of Ewing's sarcoma/PNET may lack expression of this fusion transcript.

Three types of *EWS/FLI-1* fusion transcripts have been described.[9] These transcripts vary in the amount of *EWS* and *FLI-1* exonic sequences incorporated at the site of fusion (Figure 1B). Types 2 and 3 differ from type 1 by the addition of 66 or 252 bp from *FLI-1* or *EWS*, respectively. No type 3 fusion products were detected in the series of cell lines or primary samples examined in this study. In contrast, an apparent novel fusion product that had a molecular mass slightly less than the type 2 message was detected in several patients and designated type 4 (Figure 3A, lane 3). Hybridization with junction-specific probes corresponding to the type 1 and 2 products failed to hybridize with this PCR product, confirming its unique sequence complexity (Figure 3A). Direct sequence analysis of a cloned type 4 PCR product, demonstrated an addition of 33 bp from *EWS* compared to the type 1 product (Figure 3B). This additional sequence corresponded to the 3′ terminal 33 bp from the extra

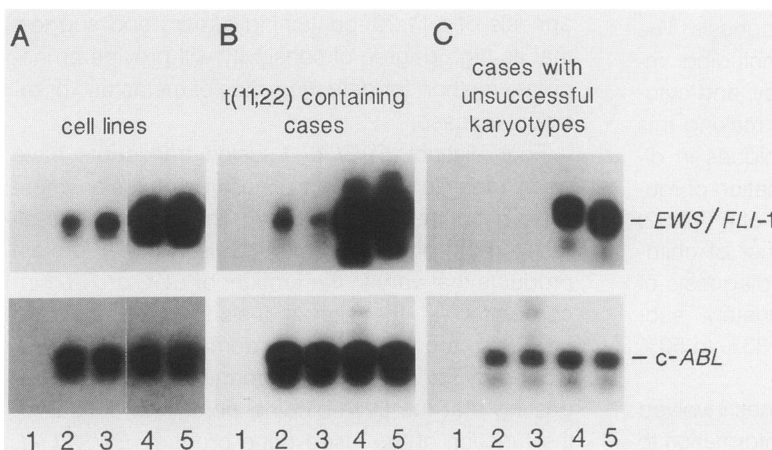
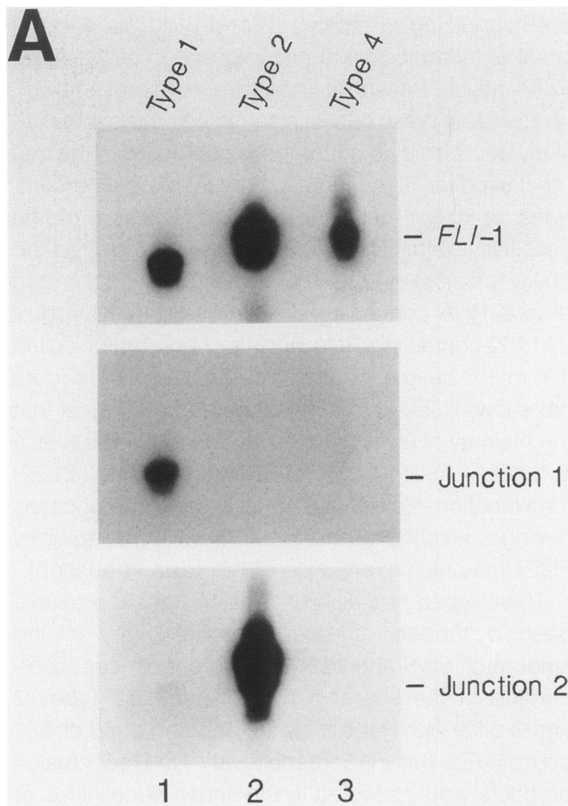


Figure 2. *Representative RT-PCR analyses of the EWS/FLI-1 fusion transcript from t(11;22)-con-taining cell lines* (**A**) *and primary clinical samples* (**B**) *or from clinical samples of Ewing's sarcoma/PNET without successful cytogenetics* (**C**). *Twenty percent of the PCR products were separated by electrophoresis in a 1.2% agarose gel after 40 rounds of amplification using the 5′A and 3′A set of oligonucleotide primers (top panel) or oligonucleotide primers for the amplification of the ubiquitously expressed message for c-ABL. The products were then transferred to nylon membranes by the method of Southern analysis, hybridized with the FLI-1- or c-ABL-specific probes, respectively, washed, and detected by autoradiography. Exposure times for autoradiography were 16 hours for the top panel and 6 hours for the bottom.*



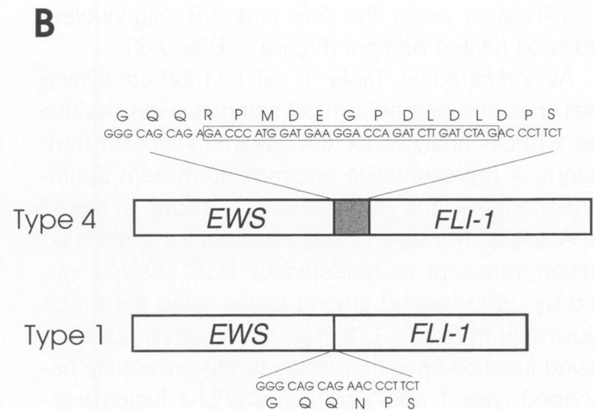


Figure 3. *Identification of the type 4 EWS/FLI-1 fusion messenger RNA.* A: *PCR products from type 1, 2, and 4 EWS/FLI-1 fusion transcripts were hybridized with the common FLI-1-specific probe (top panel) followed by stripping and rehybridization with junction-specific probes corresponding to the type 1 (5'GGGCAGCAGAACCCTTCTAT3') (middle panel) and type 2 (5'CAGCAGAGTTCACTGCTGGCCTATAA3') (bottom panel) EWS/FLI-1 fusion transcripts.* B: *Nucleic acid sequence and predicted amino acid sequence (in single letter code) of thetype 1 and type 4 EWS/FLI-1 fusion junctions.*

EWS sequence present in the type 3 fusion message[9] (Figure 1). This fusion was in-frame and resulted in a chimeric protein containing 11 additional amino acids. Rehybridization of the PCR products with junction-specific probes demonstrated a type 1 fusion in 29% of the cases, type 2 in 57%, and type 4 in 14% (Table 1).

Discussion

The accurate diagnosis of small, round cell tumors of childhood is essential for the institution of appropriate treatment and for prediction of prognosis. The development of ancillary techniques including immunohistochemistry, electron microscopy, and cytogenetics have aided the pathologist in making this decision. Foremost among these techniques in diagnosing Ewing's sarcoma is the application of routine cytogenetics.[2–7] The demonstration of a (11;22) translocation in a small, round cell tumor of childhood has become synonymous with a diagnosis of Ewing's sarcoma or PNET.[1,2] Unfortunately, successful karyotypes are obtained in only 30% to 50% of cases analyzed.[7,8]

The recent characterization of the genes involved in t(11;22) has provided the necessary information to develop RT-PCR approaches for the accurate and sensitive detection of the fusion transcript resulting from this translocation.[9] Using a previously described RT-PCR approach, we now demonstrate the presence of the *EWS/FLI-1* fusion transcript in 87% of the cases analyzed, including 18 of 18 cases of karyotypically proven t(11;22)-containing Ewing's sarcoma/PNET and 10 of 14 cases of Ewing's sarcoma/PNET without successful karyotypes. Analysis of the sensitivity of the RT-PCR analysis demonstrated the ability to detect this fusion transcript from as little as one tumor cell in 10^4 to 10^5 cells. These data demonstrate the usefulness of this RT-PCR analysis for the primary diagnosis of t(11;22)-containing tumors and suggest that its high degree of sensitivity will provide an accurate method for detecting sites of metastatic or residual disease.

Four distinct *EWS/FLI-1* fusion transcripts have been identified, including the type 4 fusion message described in this study.[9] These messages all result in the production of in-frame chimeric protein products that vary in the amount of *EWS* or *FLI-1* incorporated at the site of fusion. However, in all products, the RNA binding domain of *EWS* is replaced by the DNA-binding domain from *FLI-1*, suggesting that the DNA-binding domain is critical for the function of the fused gene product, *EWS/FLI-1*. The different *EWS/FLI-1* fusion products do not

seem to correlate with histological type of tumor (Ewing's sarcoma versus PNET), tumor location (osseous versus extra-osseous), or the age of the patient. Moreover, no correlation is observed between the level of messenger RNA expressed and the type of fusion product produced. Whether biological or clinical differences result from the different *EWS/FLI-1*-encoded fusion products or the varied levels of expression will require additional study.

The identification of four histologically diagnosed cases of Ewing's sarcoma that lack expression of the *EWS/FLI-1* fusion message is consistent with results obtained by cytogenetics analyses.[3–7] In a recently published series, only 88% of the successfully karyotyped cases of Ewing's sarcoma/PNET had a (11;22) translocation, whereas an additional 4% of the cases had a translocation of 22q12 with a chromosome other than 11.[4] Although the four negative cases in our study had unsuccessful cytogenetics, their lack of expression of *EWS/FLI-1* fusion transcript suggests that they do not have a (11;22) translocation. Insufficient material was available on these cases to determine whether the *EWS* or *FLI-1* genes were disrupted at the genomic level. The biological and clinical relationship between the classical t(11;22)-containing Ewing's sarcoma/PNET and these rare cases lacking this translocation remains to be defined. Clearly, experience from the study of leukemia-associated translocations illustrates that different translocations resulting in distinct transforming products can result in similar if not identical leukemic phenotypes.[21–23] Identification of the biological function of the *EWS/FLI-1* fusion product and its comparison with the products resulting from variant translocations should help in defining the role these genes have in the transformation process.

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