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In situ reverse-transcriptase polymerase chain reaction demonstration of the *EWS/FLI-1* fusion transcript in Ewing's sarcomas and peripheral primitive neuroectodermal tumors

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Abstract It is now widely accepted that the *EWS/FLI-1* fusion transcript is associated with tumors of the Ewing family. To test whether it is possible to detect the fusion transcript by means of combining polymerase chain reaction (PCR) methodology and immunohistochemistry, we investigated tumors of the Ewing family using in situ reverse transcriptase (RT)-PCR. We were able to demonstrate the t(11;22) fusion transcript in five of six cases of Ewing's sarcoma and four of four peripheral primitive neuroectodermal tumors. These results were confirmed using fluorescence in situ hybridization in seven tumor samples. In situ RT-PCR-labeled fusion transcripts were found in virtually all tumor cells within a given sample, indicating that each cell possessed the t(11;22) transcript. We conclude from these results that in situ RT-PCR can be used for the rapid detection of *EWS/FLI-1* fusion transcripts in biopsy material. The findings also suggest that all cells of the tumors of the Ewing family carry the *EWS/FLI-1* fusion transcript.

Keywords Small round cell tumors · Ewing's sarcoma · Translocation · Immunohistochemistry · Differential diagnosis · RT-PCR

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Introduction

Small round blue cell tumors of childhood are a heterogeneous group of neoplasms, which includes Ewing's sarcomas (ES), peripheral primitive neuroectodermal tumors (pPNET), rhabdomyosarcomas, neuroblastomas, nephroblastomas, and lymphomas. Each of these lesions may consist of uniform small round cells lacking most features of differentiation [4, 21, 27]. Because specialized treatment protocols have been initiated, it is important to be able to distinguish between these neoplasms. Ancillary techniques, such as immunohistochemistry, have proven very useful. Immunohistochemical results are often difficult to interpret, however, due to aberrant protein expression or lack of immunohistochemical reactivity, for example.

ES and pPNET account for 2% of cases of childhood cancer [12]. ES is thought to be the most undifferentiated tumor in a spectrum of neoplasms also comprising atypical ES and pPNET [5, 21]. Approximately 85% of the tumors of this group are now known to harbor the translocation t(11;22)(q24;q12), which fuses the *EWS* gene on chromosome 22 to the *FLI-1* gene on chromosome 11 [1, 6, 29]. Recently described translocations fuse the *EWS* gene to the *ERG* gene [22], the *ETV1* gene [24], the *E1AF* gene [13], or the *FEV* gene [19]. All of these fusion partners of the *EWS* gene belong to the *ETS* gene family of transcription factors [7]. The translocation event abolishes their physiological functions and instead initiates transforming activity [15, 18]. It is unclear, however, whether these translocations are the first event in malignant transformation. If so, every tumor cell would bear this translocation and would express a hybrid *EWS*/*ETS* transcription factor.

Detection of these translocations at the cytogenetic and molecular level by means of reverse-transcriptase polymerase chain reaction (RT-PCR) analysis is now a widely used diagnostic tool [2, 10]. Because karyotyping is not always possible, there is further need for simple and reliable methods for detecting such chromosomal translocations. The most sensitive technique for detect-

Table 1 Clinicopathologic features and results of reverse-transcriptase polymerase chain reaction (RT-PCR), in situ RT-PCR, and fluorescence in situ hybridization (FISH) analysis in 13 patients with Ewing's sarcoma (ES), peripheral primitive neuroectodermal tumors (pPNET), and other neoplasms. *n.d.* not determined; *n.i.* not informative

Case no.	Age at diagnosis	Gender	Diagnosis	Conventional RT-PCR t(11;22)	In situ RT-PCR G3PDH	In situ RT-PCR t(11;22)	FISH rearrangement 22q12
1	12 years	Male	ES, atypical	n.d.	+	–	+
2	16 years 7 months	Female	pPNET	+	+	+	n.i.
3	18 years 2 months	Male	pPNET	n.d.	+	+	n.i.
4	18 years 7 months	Male	ES, atypical	n.d.	+	+	+
5	47 years 11 months	Female	ES, atypical	+	+	+	+
6	6 years 7 months	Female	pPNET	n.d.	+	+	+
7	13 years 1 months	Female	ES, atypical	n.d.	+	+	+
8	13 years 4 months	Male	ES, classical	+	+	+	+
9	17 years 4 months	Female	pPNET	n.d.	+	+	n.i.
10	12 years 11 moonths	Female	ES, atypical	n.d.	+	+	+
11	1 year 3 months	Male	Neuroblastoma	n.d.	+	–	n.d.
12	1 year 8 months	Female	Wilm's tumor	n.d.	+	–	n.d.
13	14 years 10 months	Male	Embryonal rhabdomyosarcoma	n.d.	+	–	n.d.

ing particular RNA sequences, RT-PCR, is often hampered by contamination and may thus generate false-positive results. Furthermore, it is sometimes difficult to extract RNA from very small formalin-fixed, paraffin-embedded biopsy specimens. To overcome these problems and to define the cells bearing a chromosomal translocation, we employed the in situ RT-PCR technique [20], which enables the specific detection of chimeric transcripts in histologic tissue sections. This technique yields molecular data on single cells and allows a morphological evaluation at the same time.

Materials and methods

Fresh-frozen and paraffin-embedded tumor samples were retrieved from the files of the Kiel Pediatric Tumor Registry. Six ES and four pPNET were investigated for the presence of t(11;22)-encoded chimeric transcripts. The study additionally included one neuroblastoma, one embryonal rhabdomyosarcoma, and one Wilm's tumor. Patient characteristics are listed in Table 1. All tumor samples were diagnosed according to internationally accepted criteria. Subtypes are the classical and atypical ES and pPNET [21]. The tumors were routinely stained with hematoxylin and eosin, Giemsa, and periodic acid-Schiff (PAS) and immunohistochemically labeled with the markers O13 (diluted 1:5, MIC2 antigen, Signet Laboratories, Dedham, Mass.), anti-S100 (diluted 1:500, Dako, Hamburg, Germany), and anti-neuron-specific enolase (diluted 1:200, Dako) using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique [3].

RT-PCR

Frozen tumor tissue was available from three patients (Table 1). Tissue samples were homogenized and RNA was isolated according to standard protocols with Trizol (Gibco BRL, Karlsruhe, Germany). Total RNA (1 µg) was used in a one-cap RT-PCR with the EZ rTth RNA PCR Kit (Perkin Elmer, Langen, Germany). The reaction mixture consisted of reaction buffer (50 µM bicine, pH 8.5, 115 µM KOAc, 8% w/v glycerol), 3.5 mM $Mn(OAc)_2$, 300 µm each of the four dNTPs, 5 U rTth-polymerase, and 0.4 µM of each primer. Primer sequences for t(11;22) were 5'EWS: tcctacagccaagctccaagtc and 3'FLI-1: gcttccagggttggctagg. As a positive control, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was amplified in parallel using the same conditions. The primer sequences 5'G3: acgaccactttgtcaagctcat and 3'G3: ggtactttattgatggtacatg were used. Cycling parameters consisted of 65°C for 30 min, 95°C for 2 min and 40 cycles of a two-step PCR with an annealing temperature of 55°C. A DNA Thermal Cycler 480 (Perkin Elmer) was used for PCR experiments.

In situ RT-PCR

The amplification protocol of Peters et al. [20] was used with some modifications. Serial sections from each tissue block were cut onto the silanized slides provided with the Gene Amp In Situ PCR System 1000 (Perkin Elmer). The slides were allowed to dry overnight and baked at 60°C for several hours in a clean environment. They were rinsed 3× (10 min each rinse) in xylene followed by rehydration in decreasing concentrations of ethanol. Protease digestion was performed with pepsin (Boehringer Mannheim, Germany) at a concentration of 0.01% w/v in 0.1 N HCl for 2–10 min at 37°C and stopped by repeated washings in phosphate-buffered saline (PBS).

Cycling conditions, primers, and reaction mixes were as described for the in vitro RT-PCR for 35 cycles. Each specimen was investigated for the presence of t(11;22) mRNA and G3PDH mRNA. A gene amp in situ PCR system 1000 (Perkin Elmer) was used as a thermocycler. After PCR amplification, the amplified cDNA was detected using in situ hybridization with a digoxigenin-tailed oligonucleotide probe. For the t(11;22) mRNA, the oligonucleotide 5' agccaacagagcagcagctacggg 3', located 27 nucleotides downstream from the 5' EWS primer, was used as a probe. For the G3PDH mRNA, the hybridization probe was 5' agactggctcttaaaaagtgcagg 3'. The tissue samples were overlaid with hybridization buffer [50% formamide, 2× concentrated standard saline citrate (2×SSC, 300 mM NaCl, 30 mM sodium citrate), 10% dextran sulphate, and a 5 pmol/50 µl oligonucleotide probe], denatured at 94°C for 5 min, and hybridized over night at room temperature. Posthybridization washings were done twice for 15 min at 40°C with 2×SSC and 0.5% Tween 20. The immunoenzymatic detection procedure was carried out according to the DIG system user's manual (Boehringer Mannheim) with anti-digoxigenin-$F_{(ab)}$-alkaline phosphatase (1:500 in blocking buffer) and nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) as the chromogen.

Several slides were used as negative controls for each in situ RT-PCR experiment. One slide was treated with crude RNAse (Boehringer Mannheim) overnight (250 U per section diluted in

PBS at 37°C) before cycling. The other slides were amplified with primers omitted or primer pairs specific for G3PDH mRNA and detected with *EWS* gene-specific oligonucleotide probes or vice versa.

Fluorescence in situ hybridization

To facilitate the evaluation of fluorescence in situ hybridization (FISH) experiments, 50-µm thick sections of paraffin-embedded tumor samples were disaggregated [25]. Isolated nuclei were spun onto silanized glass slides, fixated in 4% paraformaldehyde in PBS, and dried. Pretreatment consisted of 1 M sodium thiocyanate (NaSCN, Sigma, Munich, Germany) at 80°C for 3 min followed by digestion with 0.2% Proteinase K (Boehringer Mannheim) in PBS for up to 10 min. Two cosmids, G9 and F7 [6], located proximal and distal to the breakpoint region EWSR1, were used for the identification of 22q12 rearrangements [9, 30]. Double-target in situ hybridizations were done according to protocols described by Stock et al. [25]. Briefly, DNA probes were labeled with digoxigenin-dUTP or biotin-dUTP (both Boehringer Mannheim) with nick-translation (Gibco BRL). Labeled probes were precipitated with human Cot-1 DNA (Gibco BRL) and sonicated salmon sperm DNA (Sigma). The DNA pellet was dissolved in 10 µl hybridization mixture containing 5 µl deionized formamide, 1×SSC, and 10% dextran sulphate. The specimens were denatured in 70% formamide/2×SSC for 4 min at 75°C and dehydrated in cold ethanol. The hybridization mixture with the probe was denatured for 5 min at 75°C and pre-annealed for 10 min at 37°C. Hybridization, probe detection, and counterstaining were performed as described by Hattinger et al. [11].

Results

All ES and pPNET reacted immunohistochemically with the antibody O13 and, to a varying degree, with the neural markers S100 and neuron-specific enolase.

To ensure primer specificity, RT-PCR reactions with total RNA from fresh tumor samples were analyzed using primers specific for the chimeric RNA of t(11;22). The primers were chosen to flank all described exon/exon combinations. Theoretically, the product length should vary between 110 bp for the combination *EWS* exon 7/*FLI-1* exon 8 and 552 bp for the combination *EWS* exon 10/*FLI-1* exon 5. As shown in Fig. 1, agarose gel electrophoresis yielded amplification products of 300 bp and 552 bp, locating the translocation breakpoint in the three samples tested between *EWS* exon 7/*FLI-1* exon 5 and *EWS* exon 10/*FLI-1* exon 5.

For in situ RT-PCR experiments, tissue permeabilization was first optimized for every tissue block using different periods of pepsin pretreatment. The intensity of the signal was strongly dependent on the degree of proteolytic digestion. Omission of pretreatment resulted in the absence of a signal. With increasing proteolysis the signal intensity increased. The optimum proteolytic digestion varied from tissue block to tissue block. It was in the range of 2–10 min. Longer digestion times destroyed the cellular architecture too highly, resulting in the loss of morphology and the inability to retain the amplified cDNA in the cytoplasm.

To determine whether in situ RT-PCR is able to detect specific transcripts and to check each tissue block for amplifiable RNA, we tested for the ubiquitously expressed G3PDH mRNA. This resulted in strong cytoplasmic staining of almost all cells in every tissue investigated (Fig. 2). With in situ RT-PCR, five of six ES and four of four pPNET displayed reactivity with primers against the t(11;22) chimeric transcripts. In positive cases, the signal was found in the cytoplasm of nearly all tumor cells, with the nucleus remaining unstained. The signal intensity did not vary between individual tumor cells and was less strong than that of the G3PDH mRNA signal.

All negative controls failed to show any positive signal. Endothelial cells and stromal cells were negative when tested for the fusion transcripts. In addition, biopsy specimens from one patient with neuroblastoma, one patient with embryonal rhabdomyosarcoma, and one patient with a Wilm's tumor proved to be totally negative.

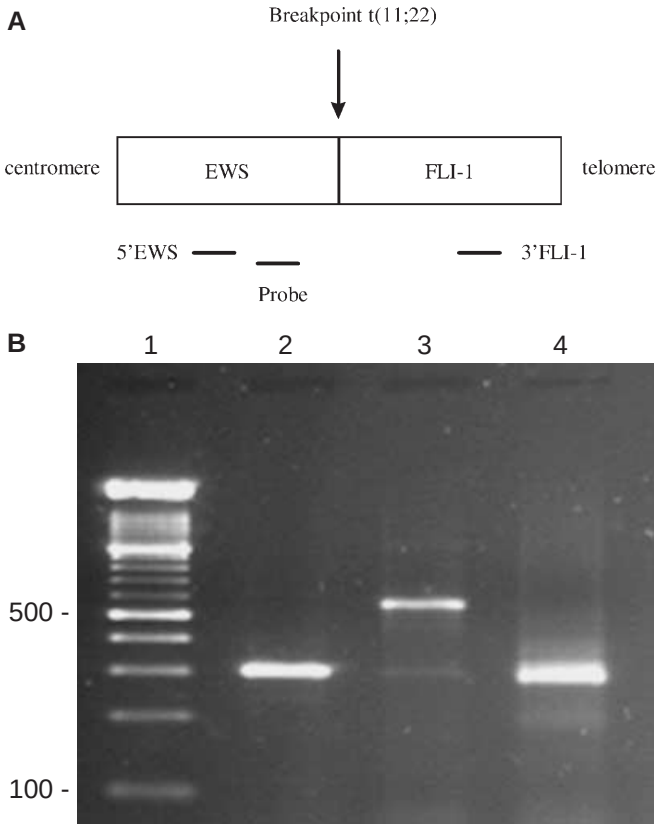

Fig. 1 **A** Position of primers and hybridization probe in relation to the hybrid gene *EWS/FLI-1*. The length of the amplification product depends on the exon/exon combination of both genes. **B** Agarose gel electrophoresis of reverse transcriptase polymerase chain reaction (RT-PCR) products with primers for t(11;22) mRNA. *Lane 1* molecular weight marker XIV (Boehringer Mannheim); *lane 2* case no. 2; *lane 3* case no. 8; and *lane 4* case no. 5

FISH

Interphase cytogenetics on isolated nuclei revealed a rearrangement of chromosome 22q12 in seven tumor sam-

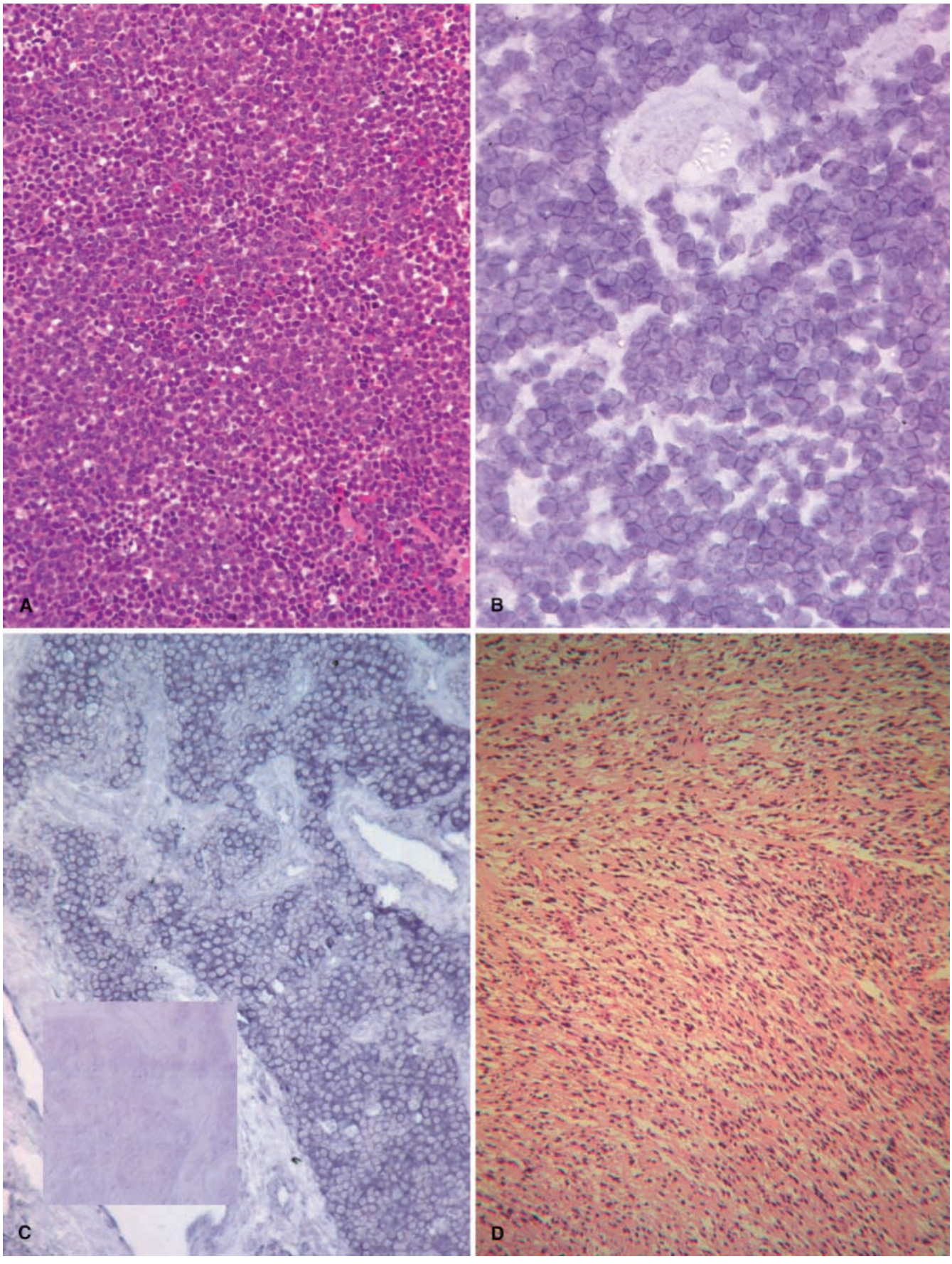

Fig. 2A–D

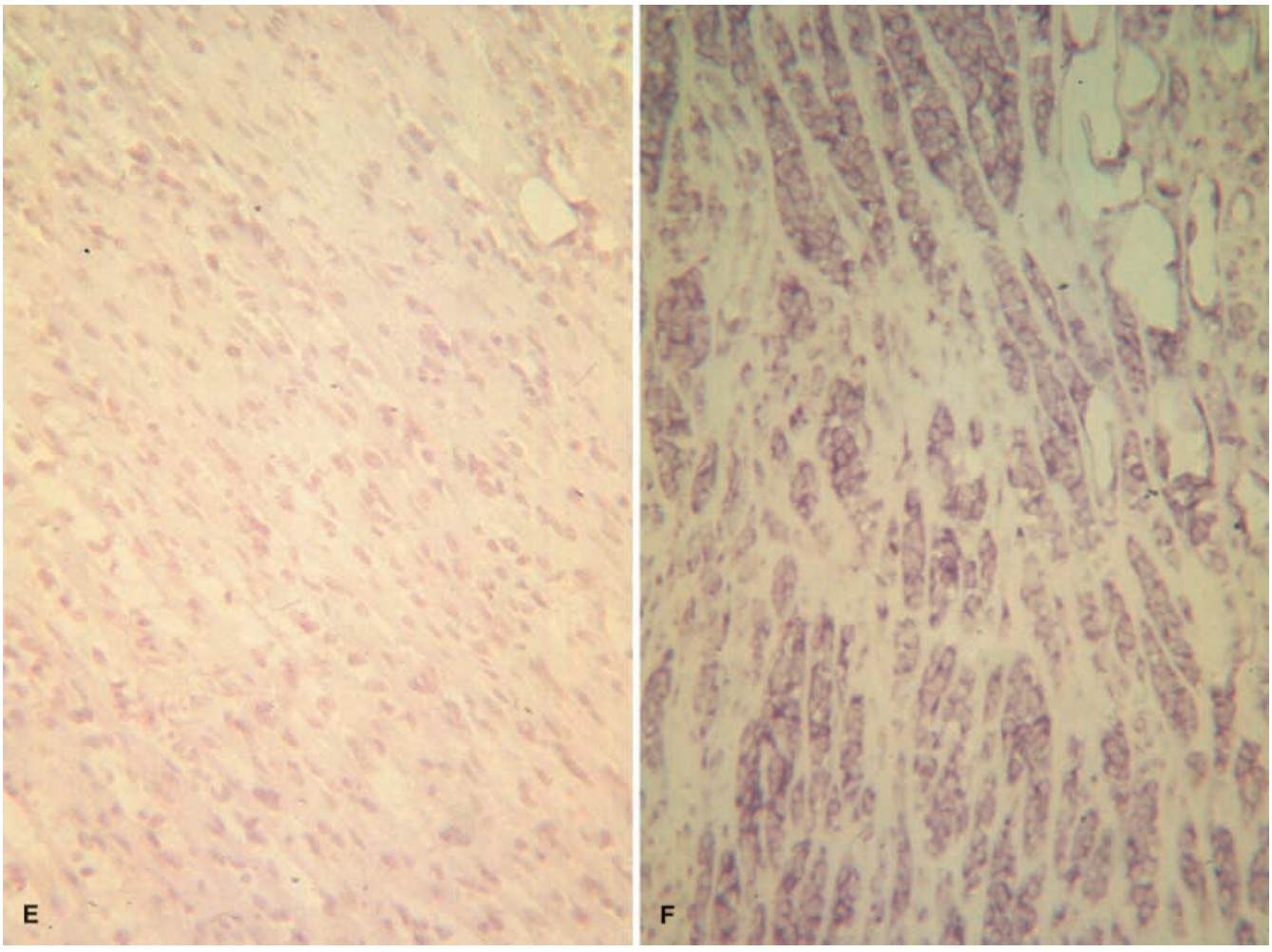

Fig. 2 Demonstration of chimeric transcripts in routine histologic specimens by means of in situ reverse transcriptase polymerase chain reaction (RT-PCR). **A–C** Atypical Ewing's sarcoma (ES), case no. 5. **A** Hematoxylin and eosin (H&E) stain (×91), **B** t(11;22) mRNA (×364); almost all tumor cells show cytoplasmic staining, whereas endothelial cells are negative. This is in contrast to (C) G3PDH mRNA (×182), where all tumor cells and endothelial cells are positive. *Insert*: omission of RT-PCR primers as a negative control. **D–F** Embryonal rhabdomyosarcoma, case no. 13. **D** H&E (×91); **E** t(11;22) mRNA (×182), no reactivity; **F** G3PDH mRNA(×182), where all cells display strong reactivity

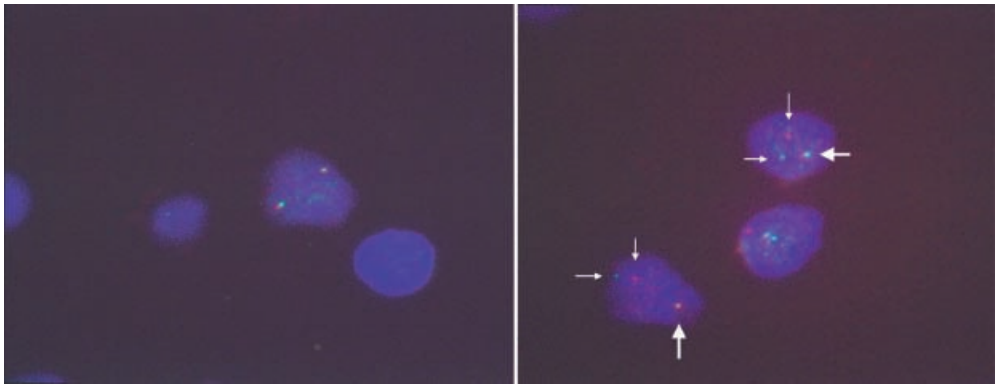

Fig. 3 Fluorescence in situ hybridization (FISH) analysis on isolated nuclei from a case of atypical Ewing's sarcoma (ES), case no. 5, with cosmid probes enclosing the breakpoint region *EWSR1*. *Left*: one normal nucleus. The *red* and *green signals* are in close proximity on both chromosomes 22, appearing as a *yellow spot* on one allele. *Right*: three tumor nuclei. Each nucleus shows a split signal (*thin arrows*) and a normal chromosome 22 (*thick arrow*), indicating a monoallelic rearrangement of chromosome 22q12

ples (Table 1). Three samples were not informative. Figure 3 shows the FISH analysis of case 5, revealing one normal nucleus and three tumor nuclei.

Discussion

The discovery of chromosomal translocations in human tumors has stirred great interest in their possible role as diagnostic or prognostic factors. The translocation (11;22)(q24;q12), first described by Aurias et al. [1] and Turc-Carel et al. [1, 28], is now considered to be specific to tumors of the Ewing family [17, 23]. For technical reasons, classical cytogenetic techniques, which were originally used to detect chromosomal aberrations, are successful in only a limited number of soft tissue sarcomas. The molecular characterization of translocations and of the genes involved led to the development of PCR-based assays, which are now widely used in diagnostic procedures. RT-PCR is the most sensitive technique for detecting chromosomal translocations in small biopsy specimens. However, because of the extreme amplification of selected gene material, care has to be taken not to generate false-positive results. Another approach for detecting chromosomal translocations is FISH [8, 14, 16]. Using DNA probes labeled with different fluorochromes, the close proximity of two signals that are normally located proximal and distal to a chromosomal break region can be demonstrated.

Although all of the techniques described above enable the accurate detection of specific chromosomal translocations, they do not allow the simultaneous morphological identification of the cells bearing the translocation. For this purpose, we developed an in situ RT-PCR for the *EWS/FLI-1* fusion transcript. Using this method, cytoplasmic reactivity was found in all but one of the Ewing's sarcomas and pPNET. Non-ES tumors were consistently negative. There was no difference in the intensity of the signal between classical ES, atypical ES, and pPNET. There was also no difference in signal intensity between individual tumor cells. The amount of positive cells was comparable to that of in situ RT-PCR for G3PDH mRNA, which was used as a control reaction. Although the abundance of chimeric transcripts is known to vary from cell to cell, depending on the cell's proliferation status [26], RNA amplification using in situ RT-PCR probably masks these differences. The reliability of our results was further supported by data gained by means of FISH analysis. Using probes encompassing the breakpoint on chromosome 22, we found a 22q12 rearrangement in all ES tumors.

Because one ES was negative when tested for t(11;22) transcripts but showed amplifiable G3PDH mRNA, it is likely that this tumor had a variant translocation partner, e.g., the *ERG* gene on chromosome 21 [22].

The data above strongly suggest that our results reflect the molecular cytogenetic status because the primers specifically amplified the target RNA in situ, e.g., on a histologic section. We adapted the protocol for use on formalin-fixed, paraffin-embedded tissue samples that had already been subjected to routine histologic examination. To overcome the problem of cross-linking of proteins due to the formalin fixation, optimum tissue pretreatment had to be determined as a first step for every tissue block. Due to the removal of some of the cytoplasmic proteins, a long digestion period probably allowed the rTth polymerase better accessibility to the RNA, thus generating stronger signals. This, however, was at the expense of tissue morphology. Furthermore, nucleic acids degraded with time, and this resulted in shortened RNA fragments. If primers that amplified relatively short regions were chosen, the probability of finding intact RNA molecules increased, thus decreasing the amount of "unreactive" tissue samples.

Since the first reports on successful in situ PCR, it is clear that such results must be interpreted cautiously and that several criteria must be met by every experiment. To ensure the specific detection of the desired sequence, we used a labeled oligonucleotide probe for hybridization with the amplified cDNA. The amplification of genomic DNA was prevented using primers that spanned several introns of the genes involved. Amplification of genomic DNA would yield too long of an amplification product and would generate nuclear signals. However, in some instances, we observed a single dot of staining in the nucleus, which probably represents the result of asymmetric and thus linear PCR amplification of the *EWS* gene. This was especially the case when tissue digestion was too harsh and the reaction components gained access to the genomic DNA. For each experiment, several negative controls were run in parallel. These included RNA digestion prior to RT-PCR, omission of primers, and the use of irrelevant primers. Non-neoplastic cells, e.g., endothelial cells, lymphocytes, and stromal cells, functioned as an internal negative control. The amplification of the ubiquitously transcribed *G3PDH* mRNA yielded a positive reaction in all cells and served as a positive control.

Because the rearrangement of 22q12 is consistently found in tumors of the Ewing family, it may be an important step in tumorigenesis [6, 15]. The transforming activity of the resulting hybrid *EWS/ETS* transcription factor is another argument in support of this hypothesis. If the rearrangement of 22q12 and the formation of a hybrid transcription factor are indeed important for tumor development, it should be demonstrable in all tumor cells. As this was the case in the positive ES and pPNET, it appears that the cells of an ES or a pPNET are identical with regard to the presence of the *EWS/FLI-1* fusion transcript. Whether this is an indication of monoclonality remains to be established.

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