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Specific and sensitive detection of the EWS/FLI1 fusion protein in Ewing's sarcoma by Western blotting

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Abstract We applied Western blotting, using an antibody against the carboxy terminal of the FLI1 protein, for detection of the 68-kDa EWS/FLI1 fusion protein in cultured Ewing's sarcoma cells and in four surgical biopsies of Ewing's sarcoma. Of six different human cell lines, the 68-kDa fusion protein was identified only in Ewing's sarcoma cells carrying the t(11;22)(q24;q12) translocation. The four samples from Ewing's sarcoma patients were also found to contain the 68-kDa fusion protein. The lowest detection level for total protein loaded on the gel was 0.3 μg. When whole Ewing's sarcoma cells were used for Western blotting without prior protein extraction, the lowest detection level was 1,300 cells. It will be possible to use Western blotting for detection of the EWS/FLI1 fusion protein in the diagnosis of Ewing's sarcoma in surgical biopsy specimens, and possibly also in fine-needle aspirates. As the method is not dependent on the quality of mRNA in the sample and involves no risk of contamination, it will be a powerful complement to the reverse-transcription polymerase chain reaction (RT-PCR).

Key words Western blotting · Fusion protein · FLI1 · Ewing's sarcoma

Introduction

The morphological diagnosis of Ewing tumour (ET: Ewing's sarcoma and primitive neuroectodermal tumour, or PNET) on the basis of biopsies or fine-needle aspirates is often very difficult [2, 10, 11]. ET is recognized histopathologically as a tumour composed of small round cells, and it can be confused with other small-cell tumours, including lymphoma, neuroblastoma, rhabdomyosarcoma or Merkel cell tumour, or with benign conditions, such as osteomyelitis [14–17]. ET is seen mainly in childhood, and in this age group it accounts for approximately 24% of all malignancies, or 29 per million children [16]. Since ET is so primitive there are no structural or ultrastructural, enzymatic, or cell surface characteristics that are specific for this entity. Diagnosis is frequently made, therefore, by excluding other differential diagnoses [15]. Over the last few years several ET-specific translocations have been discovered. Ninety percent of ET cases carry the translocation t(11;22)(q24;q12) [5, 18, 22], 5% carry t(21;22)(q22;q12) [12], and <5% carry t(7;22)(p22;q12) [7]. Analysis of translocations by reverse transcription-polymerase chain reaction (RT-PCR) is a useful option in the diagnosis of ET [3, 4, 9]. One limitation of RT-PCR in surgical pathology is the requirement for careful and rapid handling of fresh material to avoid degradation of tumour cell mRNA. The possibility of simply analysing the product of the *EWS/FLI1* fusion gene would therefore provide an additional investigation, especially if no material suitable for RT-PCR is available.

In this study we investigated whether the EWS/FLI1 fusion protein can be detected by Western blotting. This application would be a powerful complement to RT-PCR in the diagnosis of Ewing's sarcoma and PNET.

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Materials and methods

The Ewing's sarcoma cell line HTB-166 *(EWS/FLI1)*, the breast cancer cell line MDA 231, the human colonic carcinoma cell line WiDr, and the human melanoma cell line SK-MEL-2 were obtained from the American Type Culture Collection, USA. TTC-466 and TTC-633, both of which carry the t(21,22)(q22;q12) *(EWS/ERG)* translocation, were kindly provided by Dr. P. Sorensen, BC Research Institute for Child and Family Health, Vancouver, Canada. Simian virus-40-transformed human fibroblasts (line 90VAVI) were kindly given to us by Dr. G. Stein (University of Colorado, Boulder, Colo., USA). The synovial sarcoma cell line A-3243 was kindly provided by Dr. S.A. Aaronson (Rockefeller Institute, New York, USA).

The MDA-231 cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, glutamine, sodium pyruvate, bensylpenicillin and streptomycin. HTB-166 was cultured in RPMI-1640 containing 10% fetal calf serum. All other cell lines were cultured in minimum essential medium supplemented with 10% fetal calf serum and with the addition of nonessential amino acids.

Cells were grown in monolayers in tissue culture flasks with a 95% air/5% CO_2 atmosphere maintained at 37°C in a humidified incubator. For experimental purposes the cells were cultured in 15-cm dishes. Cells were seeded at a density of 3,000–5,000 cells/cm2 and harvested when they had reached subconfluency.

C-19 is a rabbit polyclonal IgG antibody raised against a peptide corresponding to amino acids 434–452 mapping at the carboxy terminus of the FLI1 protein. The epitope is localized closer to the C-terminus than to the ETS-binding domain, which is essential for the binding of the transcription protein to DNA [19]. The peptide (sc-356P) from which C-19 was raised was used to confirm the specificity of the antibody. In this experiment the control peptide was mixed with the antibody solution. The concentration (w/v) of the peptide was ten-fold that of C-19. The secondary antibody was goat anti-rabbit IgG-HRP (sc-2004). C-19, the peptide and the secondary antibody were all purchased from Santa Cruz Biotechnology (USA) through Scandinavian Diagnosis Services (Falkenberg, Sweden).

Biopsies from four cases of Ewing's sarcoma were used for Western blotting. It had been verified by cytogenetic analysis that these tumours were carrying the t(11;22)(q24;q12) translocation. Furthermore, all four cases exhibited a characteristic histology for ET, and were strongly positive for MIC-2. The non-ET samples studied were biopsies of a high-grade malignant fibrous histiocytoma, a dermatofibrosarcoma protuberans and a childhood neuroblastoma.

Total cell protein was isolated as described elsewhere [6]. Cells were harvested and homogenized in a buffer containing 0.32 M sucrose, 1 mM taurodeoxycholic acid, 2 mM $MgCl_2$, 1 mM EDTA, 25 mM benzamidine, 1 μg/ml bacitracin, 2 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml soya bean trypsin inhibitor and 10 μg/ml leupeptin. After 10-min centrifugation at 600 *g* (4°C) the pellet (containing unbroken cells and cytoskeleton) was discarded. The supernatant was used for analysis. The concentration of total protein was measured using the Bio-Rad protein assay (Bio-Rad, Germany) according to the method of Bradford [1]. Tumour samples were washed twice with PBS, and weighed. Half of each sample was homogenized for protein isolation and Western blotting.

For SDS-PAGE studies, protein and cell samples were dissolved in a buffer containing 0.0625 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, bromophenol blue and dithiothreitol (DTT). Different amounts of the samples were analysed by SDS-PAGE with a 4% stacking gel and a 10% separation gel at 80 V overnight, essentially according to the protocol of Laemmli [8]. The 5x running buffer was prepared by mixing Tris base, glycine and SDS in 1 l distilled water, pH 8.3; 20 μl SeeBlue Pre-Stained Standard (NOVEX, San Diego, USA) was run simultaneously. Three SDS-PAGE gels were run for each experiment, and one was stained with Coomassie Blue to monitor the quality of the proteins.

After SDS-PAGE the separated proteins were transferred at 100 V for 3 h to a Hybond-ECL nitrocellulose membrane (Amersham Life Science, Amersham, Bucks. UK) for Western blotting. The transfer buffer was prepared by mixing 12.15 g Tris base, 56.25 g glycine and 1 l methanol in 5 l dH_2O. The membranes were subsequently blocked for 1 h at room temperature with a blocking solution containing 10% (w/v) skimmed milk powder and 0.3% (v/v) Tween 20 in PBS, pH 7.5. The membranes were incubated with the primary antibody (C-19) (1/500 dilution) for 1 h. After two washes, the membranes were incubated with a horseradish peroxidase-linked secondary antibody (1/1500 dilution) for 1 h. After the washes, the membranes were incubated in ECL Western blotting detection reagents (Amersham) for 1 min. The membranes were exposed to Hyperfilm-ECL for 1 min, 5 min or overnight, whereupon fluorography was performed.

For RT-PCR, the primers used were synthesized according to published sequence information [9, 20]. Total RNA was isolated using the RNazol B method as instructed by the manufacturer. The samples were first reverse-transcribed using the primer 11A. The RT solution consisted of 40 nmol of a dNTP DNA polymerization mix (Pharmacia Biotech), 20 U RNase inhibitor (Boehringer Mannheim), 10 μg albumin (MBI Fermentas), 4.0 μl 5× first-strand buffer (Gibco BRL Life Technologies), 2.0 μmol DTT, 200 U Superscript RT (Gibco BRL Life Technologies), 200 ng primer, and different amounts of the isolated RNA in a final volume of 20 μl. The reaction was performed at 42°C for 1.5 h. The resulting cDNA was amplified by PCR using primers 11.3 and 22.3. The PCR reaction solution consisted of 20 nmol dNTPs DNA polymerization mix (Pharmacia Biotech), 5 μl 10× reaction buffer for Taq DNA polymerase (MBI Fermentas), 0.125 μmol $MgCl_2$ (Gibco BRL Life Technologies), 5 μl cDNA from RT, 200 ng primers and 4 U Taq polymerase (MBI Fermentas) in a final volume of 50 μl.

Amplification (45 cycles) was performed at 94°C for 1 min, 68°C for 1 min and 72°C for 1.5 min. In all RT-PCR experiments strict precautions were undertaken to avoid cross-contamination or product carry-over. The pre- and postamplification steps were separated spatially from each other. The PCR products were run on a 1.2% TBE agarose gel and detected by ethidium bromide staining.

Results

It was first confirmed that the Ewing's sarcoma cell line (HTB-166) used in this study contained the t(11;22)(q24;12) translocation. RNA from HTB-166 cells was isolated using the RNazol B method, after which RT-PCR was performed using primers specific for the *EWS/FLI1* transcript (see "Materials and methods"). A positive PCR band (390 bp) is shown in Fig. 1. The negative control showed no bands.

To investigate the capability of Western blotting for detecting the EWS/FLI1 fusion protein isolated from Ewing sarcoma's cells, we used an antibody against the carboxy terminal of FLI1. As shown in Fig. 2, a protein with a size of approximately 68 kDa was detected for this antibody. In order to estimate the sensitivity of the method for detection of this protein, different amounts of tumour cell proteins were analysed. The lowest detection

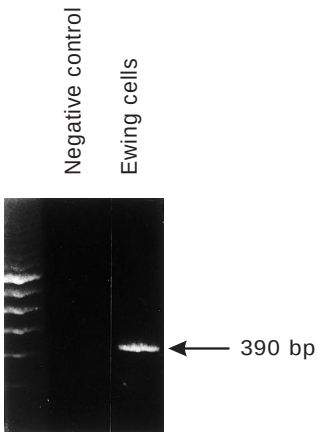


Fig. 1 Detection of *EWS/FLI1* fusion gene transcript in HTB-166 cells by the reverse-transcription polymerase chain reaction (RT-PCR). The 390-bp PCR product is shown

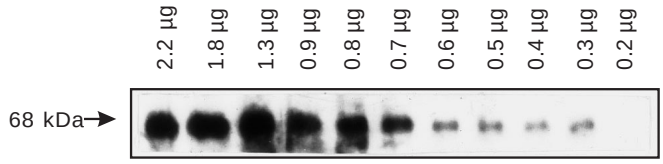
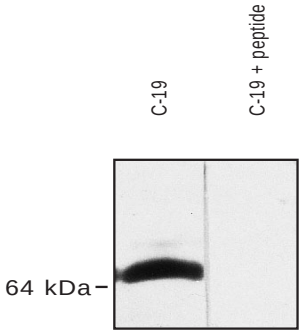


Fig. 2 Detection of the 68-kDa EWS/FLI1 fusion protein in HTB-166 cells using the FLI1 antibody (C-19). The amounts (0.2–2.2 µg) of loaded total protein are indicated

Fig. 3 Specificity of the C-19 antibody was tested using the peptide (sc-356P) from which C-19 was raised. The peptide was added at a concentration exceeding the antibody concentration ten-fold



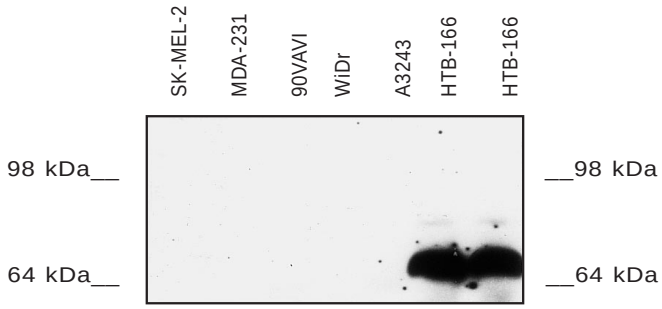


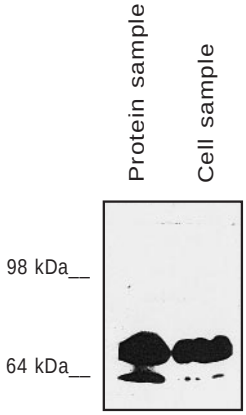
Fig. 4 Specificity of the FLI1 antibody was tested on the indicated cell lines (five non-ET and one ET): 13-µg total protein samples from each cell line were analysed by Western blotting

level was found to be as low as 0.3 µg protein. Figure 3 shows that the 68-kDa band disappeared if the control peptide (sc-356P) was added together with FLI1 antibody.

To investigate the specificity of the FLI1 antibody further, total protein from each of the cell lines HTB-166 (Ewing's sarcoma cells), SK-MEL-2 (human melanoma cells), MDA-231 (human breast cancer cells), 90VAVI (SV40-transformed human fibroblasts), WiDr (human colonic carcinoma cells) and A-3243 (human synovial sarcoma cells) was loaded on the SDS-PAGE gel. As shown, the 68-kDa protein was detected in HTB-166, but not in samples from any of the non-ET cell lines (Fig. 4). Samples from TTC-466 and TTC-633, both carrying the t(21;22)(q22;q12) *(EWS/ERG)* translocation, were also analysed. However, no positive signal was seen in these samples (data not shown). This means that the the FLI1 antibody used does not cross-react with the ERG protein.

We also investigated whether it was possible to use whole cells directly, without protein isolation, for SDS-PAGE and Western blotting aimed at detection of the fusion protein. Hence, HTB-166 cells were mixed with loading buffer and boiled at 95°C for 6 min and then loaded on the gel. As shown in Fig. 5, the 68-kDa protein was clearly detected in a sample containing 10,000 HTB-166 cells. Smaller cell samples were also tested, and we found that the lowest detection level was only 1,300 cells (data not shown).

Fig. 5 Detection of EWS/FLI1 fusion protein on whole cell samples from HTB-166: 10,000 cells were loaded onto the gel. As a control a protein sample from HTB-166 was also loaded. The FLI1 antibody was used for detection of the fusion protein



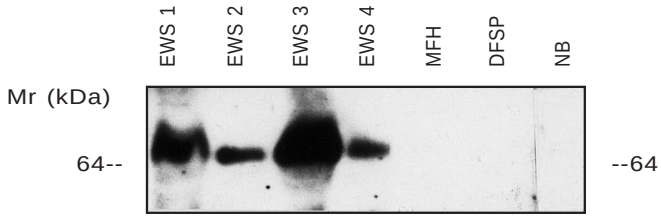


Fig. 6 Detection of EWS/FLI1 fusion protein in protein samples from four cases of Ewing's sarcoma (*EWS1–4*). The FLI1 antibody (C-19) was used for detection on the membrane. Non-ET controls were protein samples from a malignant fibrous histiocytoma (*MFH*), a dermatofibrosarcoma protuberans (*DFSP*), and a neuroblastoma (*NB*). The amounts of protein samples analysed corresponded to the following weights of tumour tissue: *EWS1*, 48 µg; *EWS2*, 29 µg; *EWS3*, 19 µg; *EWS4*, 6 µg; *MFH* 31 µg; *DFSP*, 31 µg; and *NB*, 20 µg

Finally, we analysed protein samples isolated from four surgical biopsies of Ewing's sarcomas. All tumours had been proven to carry the 11;22 translocation *(EWS/FLI1)*. As can be seen in Fig. 6, the 68-kDa fusion protein was clearly detected in all of these four Ewing's sarcoma samples. The sample amounts analysed corresponded to 6–48 µg tumour tissue. All three non-ET control samples (MFH, DFSP and NB) were negative for the 68-kDa protein (Fig. 6).

Discussion

Molecular analysis of the 11; 22 rearrangements is likely to have an impact on the diagnostic management in Ewing's sarcoma and PNET. Moreover, molecular analysis of tumour-associated gene rearrangements is an important tool that can be used in disclosing the mechanisms of oncogenesis. It seems clear that the formation of a

transcription factor from the *EWS/FLI* hybrid gene is a necessary step in tumorigenesis of ET [13, 19, 20].

We have found that the 68-kDa EWS/FLI1 fusion protein can be detected by Western blotting using an antibody (C-19) against the carboxy terminal of the FLI1 protein. The epitope for C-19 is localized downstream of the ETS domain, which is necessary for the DNA binding of the ETS family of transcription factors [19]. We can confirm that this antibody is highly specific, since the fusion protein was only detected in Ewing's sarcoma cells carrying the t(11;22)(q24;12) translocation. All other cell types studied, including cells carrying the *EWS/ERG* translocation, were negative. *ERG* is a member of the ETS family of oncogenes [19]. Western blotting using C-19 was also found to be very sensitive, since as little as 0.3 μg of the total Ewing's sarcoma cell protein was enough to detect the EWS/FLI1 fusion protein.

We also found that detection by Western blotting could be performed directly on cells without prior protein extraction. Under such conditions the detection level was as low as 1,300 cells. We also analysed frozen biopsy samples from four different Ewing tumours. A protein sample derived from 6 μg of tumour tissue was enough to give a strong band. This implies that Western blotting, without the risk of consuming much of the material for histopathological analysis, can be used for diagnostic examination of surgical biopsies. The analysis time was only 2 days.

Since Western blotting seems to be a sensitive method of detecting the EWS/FLI1 protein, this assay may also prove to be applicable to cytological samples. This option might offer an important adjunct to RT-PCR in the diagnosis of Ewing's sarcoma and PNET, especially since it is independent of contaminants and the quality of mRNA. There is also the possibility of applying immunohistochemistry for the detection of the EWS/FLI1 fusion protein on processed surgical specimens.

However, Western blotting cannot contribute to give closer information about the diversity of chimeric transcripts caused by t(11;22). Such information, which may be of prognostic relevance in ET [4, 20, 21, 22], requires molecular analysis of the tumour samples.

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