Detection of Minimal Bone Marrow Infiltration in Patients with Localized and Metastatic Ewing Sarcoma Using RT-PCR

(Ewing sarcoma / micrometastases / minimal residual disease / RT-PCR / chromosomal translocations)

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Abstract. Ewing sarcoma and related neoplasias are characterized by the presence of specific chromosomal translocations resulting in *EWS/ETS* gene rearrangements. Created EWS/ETS-oncogene fusion transcripts can be detected in up to 98% of ESFT and provide tumour-specific markers useful in diagnostics. Using RT-PCR for detection of this aberration we can reveal minimal amounts of tumour cells contaminating BM, blood or apheresis products. We have examined BM samples from 22 patients (21 newly diagnosed and one recurrent disease) with histologically confirmed ESFT for the presence of contaminating tumour cells in BM at the time of diagnosis. Sixteen patients presented with localized disease, six had distant metastases at the first presentation. Ewing sarcoma cells were detected in the BM of 5/16 (31%) patients with localized disease and 3/6 (50%) with clinically detectable metastases at diagnosis. BM smears prepared from the same aspirates evaluated by light microscopy were all negative, even in two patients with multiple bone disease. We have confirmed the high sensitivity of the RT-PCR assay for detection of minimal BM infiltration in localized and metastatic ESFT. We have found that more than a quarter of patients with localized ESFT have minimal BM infiltration. Although the clinical significance of the minimal disease detected at the molecular level remains unknown, RT-PCR evaluation may enable better stratification of patients into risk groups in the future.

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Abbreviations: BM – bone marrow, ESFT – Ewing sarcoma family of tumours, MRD – minimal residual disease, pNET – peripheral primitive neuroectodermal tumour, RT-PCR – reverse-transcriptase polymerase chain reaction.

The Ewing sarcoma family of tumours (ESFT) consists of highly malignant neoplasms of bone and soft tissues affecting mostly children and young adults. The ESFT includes classic Ewing sarcoma of bone, extraosseous Ewing sarcoma and peripheral primitive neuroectodermal tumors (pPNET) of bones or soft tissues (Horowitz et al., 1997; West 2000; Sandberg and Bridge, 2001). These malignancies share a common neural histogenesis and tumour genetics; ESFT is believed to be derived from postganglionic parasympathetic primordial cells (Horowitz et al., 1997). Although often presented as localized solid malignancy, ESFT is most probably a systemic disease with micrometastases at the time of diagnosis, as evidenced by a 10% cure rate in a historical group of patients, treated with local therapy only (Horowitz et al., 1997; de Alava and Gerald, 2000). Intensive multimodal treatment protocols combining surgery, systemic chemotherapy and radiotherapy improved event-free survival rates up to 60% (Jürgens et al., 1998). Approximately 1/4 of patients with ESFT have detectable metastatic disease at the time of diagnosis, and this group still do poorly despite intensive multimodality therapy, with 5-year overall survival below 20% (Jürgens et al., 1998; Kovar, 1998; Kushner and Meyers, 2001).

The primary genetic event in ESFT is chromosomal translocation resulting in fusion of the *EWS* gene at chromosome 22 with a member of the *ETS* gene family of transcription factors (Delattre et al., 1994; Zoubek et al., 1994; Sandberg and Bridge, 2001). The most frequent translocations are t(11;22) (q24;q12) and t(21;22) (q22;q12). The former translocation results in fusion of *EWS* to *FLI-1* (most frequent in ESFT, 90–95%), the latter generates chimaeric transcripts between *EWS* and *ERG* (5–10%), both creating new fusion proteins associated with tumour cell formation. The discovery of specific chromosomal translocations enables high sensitivity detection of tumour cells in various biologic material by means of reverse-transcriptase polymerase chain reaction (RT-PCR), which is now used in the

diagnostics of these malignancies (Peter et al., 1995; Meier et al., 1998; Zoubek et al., 1998). In rare instances of ESFT, *EWS* is fused to the *ETS* domains of *ETV-1*, t(7;22) (p22;q12), *E1AF*, t(17;22) (q22;q12) or *FEV*, t(2;22) (q33;q12) or complex translocations occur affecting 22q12 and 11q24 (de Alava and Gerald, 2000; Llombart-Bosch et al. 2000; Sandberg and Bridge, 2001). The new chimaeric EWS/ETS molecules, chimaeric fusion proteins, modulate the transcription of some specific target genes (critical target genes for EWS/ETS oncoproteins are being investigated) and these events initiate and maintain tumorigenesis in ESFT (Ohno et al., 1993; Desmaze, 1997; Kovar, 1998). In experiments evaluating the ability of fusion proteins to cause neoplastic transformation, the chimaeric molecules have been shown to affect the growth characteristics of some cell lines. Transfection of mouse fibroblasts with *EWS/FLI-1* or *EWS/ERG* can transform cells, so that they acquire tumour-like properties, such as growth in soft agar and immunodeficient mice (May et al., 2000). This and other findings suggest the key role of chimaeric proteins in the development, growth, clinical heterogeneity and behaviour of ESFT (de Alava et al., 1998; Ginsberg et al., 1999; Lin et al., 1999).

Bone marrow (BM) examination is a routine part of staging in ESFT. Positive results of cytological examination indicate an advanced-stage disease with severe consequences for patient treatment and prognosis. The frequency of BM involvement at the time of the first presentation is not precisely known and only a few reports exist about the use of RT-PCR for detection of minimal BM infiltration (Oberlin et al., 1995; Peter et al., 1995; Pfleiderer et al., 1995; Horowitz et al., 1997; Zoubek et al., 1998). The aims of this study were to verify the applicability of RT-PCR for detection of minimal BM infiltration and to investigate the incidence of BM infiltration in ESFT patients with RT-PCR.

Material and Methods

BM aspirates were obtained at the time of diagnosis from patients with histologically confirmed ES/pPNET. All samples were collected in ethylenediamine tetraacetic acid (EDTA) before starting the treatment. BM smears were evaluated by light microscopy as well. Trephine biopsies were not performed. Two distinct groups of patients were established, the first with localized disease (defined as those with no evidence of distant

Table 1. Patients' characteristics

Patient No.	Age/Sex	Histology/ mol.biology	Localization	Metastases	RT-PCR		Outcome	Follow up (month)
					No. samples	No. positive		
1	12F	ES (EWS/ERG)	ribs	reg. LN	1	0	CR1	19
2	6M	ES (EWS/ERG)	fibula	No	2	2	CR1	8
3	2M	ES (EWS/FLI-1)	fibula	No	2	0	CR1	11
4	15M	ES (EWS/FLI-1)	humerus	No	2	1	CR1	8
5	9M	ES (EWS/FLI-1)	femur	No	2	0	CR1	6
6	12F	pPNET - n.d.	head - EET	No	2	0	CR1	18
7	22M	pPNET (EWS/ERG)	scapula - EET	L, B	3	2	POD syst	10
8	12F	ES (EWS/FLI-1)	femur	No	2	0	POD loc+syst	11
9	14F	ES - n.d.	pelvis	No	1	0	CR1	23
10	12F	pPNET (EWS/ERG)	L3 - S1	L	2	0	DOC	1
11	10F	ES - n.d.	femur	No	2	1	POD loc+syst	22
12	15F	ES (EWS/FLI-1)	fibula	L	2	2	CR1	6
13	15F	ES - negative	neck -EET	L	2	0	POD syst	7
14	14F	ES (EWS/FLI-1)	pelvis	No	1	0	TRD	11
15	13F	ES (EWS/FLI-1)	pelvis	No	1	0	CR1	18
16	16F	ES - n.d.	sacrum	No	1	0	DOC	7
17	9F	pPNET (EWS/FLI-1)	kidney	No	2	2	DOC	5
18	9F	ES - n.d.	femur	No	2	1	CR1	7
19	15M	ES (EWS/FLI-1)	pelvis	L, B	2	0	POD syst	16
20	15M	ES - n.d.	humerus	LN	2	1	CR1	10
21	17M	ES (EWS/FLI-1)	femur	No	2	0	CR1	8
22	15M	ES (EWS/FLI-1)	tibiae	No	2	0	CR1	21

B – bones, CR – complete remision, DOC – died of complications, EET – extraosseous tumour, ES – Ewing sarcoma, L – lungs, LN – lymph nodes, loc – localized, n.d. – not done, POD – progressive disease, syst – systemic, TRD – transplant-related death.

metastases with imaging techniques and cytomorphologic examination of BM aspirates), and the second with known metastatic disease, who were evaluated separately. Samples were collected from twenty-two patients, 21 newly diagnosed and one relapsed, treated previously in another institution before entering our department. The snap-frozen primary tumour tissues, collected during open surgical biopsy, were available to confirm the presence of disease-specific gene rearrangement in 15 cases. Patients' characteristics including clinical and molecular data are shown in Table 1.

Total RNA was isolated from BM (10×10^6 nucleated cells per sample). RNA extraction was based on the modified guanidium isothiocyanate-phenol-chloroform method (Chomczcynski and Sacchi, 1987) using the TRIzol reagent (Gibco BRL, Life Technologies, Grand Island, NY) and subjected to reverse transcription and PCR in search of EWS/FLI-1 and EWS/ERG fusion transcripts. The assay was performed using 1–3 μg of total RNA as a template, GeneAmp RNA®PCR Core Kit (Perkin-Elmer, Foster City, NY) following the manufacturer recommendations and oligonucleotide primers originally published by Meier, 1998 (EWS1: 5'-GCAGCAGCCTCCCACTAGTTACC-3', FLI1/2: 5'-CAGGTGATACAGCTGGCGTTGG-3', ERG2: 5'-CAGGTGATGCAGCTGGAGT TGG -3'and primers for nested PCR, EWS2: 5'-CTGGATCCTACAGC-CAAGCTCC-3', FLI1/1 : 5'-CAGGAGGAATTGC-CACAGCTGG-3', ERG1: 5'-CAGGAGGAACT GCCAAAGCTGG -3'). Briefly, aliquots of total RNA were reverse-transcribed with random hexamers using Moloney murine leukaemia virus (M-MULV) reverse transcriptase. Reverse transcription included incubation at 37°C for 50 min followed by a 5 min period at 95°C to denature the enzyme. The quality of cDNA synthesis was confirmed by amplification of ubiquitous *β-actin* gene in every studied case. The first round of PCR was then performed with an initial denaturation step of 95°C for 10 min, followed by 35 cycles, including denaturation at 95°C for 30 s, annealing at 62°C for 1 min and extension at 72°C for 1 min, with a further 7 min extension step after completion of the last cycle. The second round (nested) of PCR was performed using internal primers and 1 μl of the initial PCR product. Assays were performed in a Perkin Elmer 9600 automated cycler. PCR products were analysed by 1.5% agarose gel electrophoresis with ethidium bromide staining and detected using Gel Doc 2000 TM (Bio-Rad, Hercules, CA). Positive (cell line IARC-EW2) and negative controls were carried through all PCR reactions steps. We have also evaluated BM samples obtained at the time of diagnosis from patients with other malignant solid tumours of childhood. BM samples from patients with neuroblastoma (5 patients), rhabdomyosarcoma (3), osteosarcoma (2) and synovialosarcoma (1), and samples from healthy controls (5) were all negative, thus confirming the high specificity of our method.

Results

We performed molecular detection of EWS/FLI-1 and EWS/ERG fusion transcripts in 40 samples of BM aspirates, obtained from twenty-two ESFT patients at the time of the first presentation. The primary tumour tissue was available in fifteen cases to confirm the presence of t(11;22) or t(21;22) translocations. Light microscopy evaluation of BM smears did not confirm BM involvement in any of our studied cases. Among the group of sixteen patients with localized ESFT, five were positive in BM (31%, altogether 27 samples/7 positive, see Table 2). Of six patients with clinically apparent metastatic disease at the time of diagnosis (relapse), we proved tumour cells in BM of three (50%, altogether 13 samples/6 positive).

Discussion

The stage of disease at the time of diagnosis remains the most powerful independent predictive factor of patient prognosis in ESFT. Distant metastases detected and visualized by imaging techniques or BM infiltration revealed by cytology examination place the patient into an unfavourable prognostic group. The most unfavourable prognosis is for patients with ES/pPNET metastatic to bone and/or BM (Horowitz et al., 1997; Jürgens et al., 1998; Kushner and Meyers, 2001). However, the group of patients with localized disease may contain some high-risk cases with minimal metastatic diseases invisible to these detecting tools. Previous identification of tumour cell-specific chimaeric gene transcripts in ESFT gives an opportunity for high-sensitivity detection of minimal amounts of contaminating cells in blood, BM or autologous grafts using RT-PCR (Peter et al., 1995; West et al., 1997; Zoubek et al., 1998).

In the work presented here, we were looking for minimal BM infiltration at the time of diagnosis (or disease relapse in one patient) by means of RT-PCR in patients diagnosed with ESFT. We have shown that it is possible to detect chimaeric transcripts by RT-PCR in BM in patients with ES/pPNET with high sensitivity and we confirmed the usefulness of RT-PCR for minimal residual disease (MRD) detection. In our group, 31% of patients

Table 2. Results of RT-PCR detection for the EWS/FLI-1 and EWS/ERG transcripts in BM samples of ESFT patients.

Patient group	Patients No. / Positive No.	BM samples No. / Positive No.
Localized	16 / 5 (31 %)	27 / 7 (26%)
Metastatic	6 / 3 (50 %)	13 / 6 (46%)
All	22 / 8 (36 %)	40 / 13 (33%)

Table 3. BM infiltration in Ewing sarcoma at the time of diagnosis - results of different groups

Source	Patients		Localized sarcoma		Metastatic sarcoma	
	No+/total	% positive	No+/total	% positive	No+/total	% positive
Fagnou 1998	14/43	33%	6/28	21%	8/15	53%
Zoubek 1998	16/35	46%	7/23	30%	9/12	75%
Pfleiderer 1995	6/16	38%	1/9	11%	5/7	71%
West 1997*	5/22	23%	3/16	19%	2/6	33%
Athale 2001**	7/26	27%	0/11	0%	7/15	47%
Our results	8/22	36%	5/16	31%	3/6	50%

* t(21;22) not evaluated, ** including rhabdomyosarcomas and desmoplastic small-round-cell tumours

with non-metastatic disease were RT-PCR positive for the marker mRNA in BM, a result in consent with observations (approx. 20%–30%) reported by others (Pfleiderer et al., 1995; West et al., 1997; Fagnou et al., 1998; Zoubek et al., 1998; Athale et al., 2001). Table 3 gives a summary of results published by different investigating groups to date.

Six patients in our study presented distant metastases, three in the lungs solely, one in the lymph nodes and two had lung and multiple bone metastases. We found minimal BM infiltration in only three of six patients with metastatic disease. The tumour tissue sample was available in five cases with advanced disease. In a 15-year-old girl (case 13) with systemically relapsed soft tissue ESFT arising in her neck, we were unable to confirm neither t(11;22), nor t(21;22) in the primary tumour. This particular patient should therefore be excluded from our final analysis. The failure to detect EWS/FLI-1 or EWS/ERG mRNA in this case can be explained in several ways. It may contain another rare alternative EWS/ETS rearrangement not tested in our study – (t(7;22), t(17;22) or t(2;22)), or previous chemotherapy and local radiotherapy led to neural differentiation and absence of detectable *EWS/ETS* gene expression (West et al., 1997; Knezevich et al., 1998). In the four remaining cases, two had *EWS/ERG* rearrangements documented in the tumour tissue, and two patients had multiple bone metastases without BM involvement in light microscopy evaluation. The presence of tumour cells with t(21;22) in BM had been documented in one case with RT-PCR. The BM of the second patient (case 19) was RT-PCR negative. This finding is in contrast to the results published by Zoubek et al. (1998), who reported RT-PCR positivity in BM for all five patients with bone metastases and for 50% of patients with lung metastases. West et al. (1997) found two of six patients with metastatic disease positive in BM and 5 of 10 positive in peripheral blood, which he interpreted as 50% presence of micrometastases in their group of patients with advanced disease. Unfortunately, no information on metastatic sites was given, so that comparison between the results is impossible like in other groups.

Despite the given findings, it should be noted that there are many factors which can affect the RT-PCR analysis and the correct interpretation of results. Possible factors affecting analysis are: sampling errors due to inappropriate anticoagulants used, excessive BM dilution by blood, under-sampling due to the focal BM involvement in ESFT, and others (Kovar, 1998).

During every evaluation, strict precautions were taken to avoid cross-contamination, pre- and post-amplification steps were separated from each other, negative and positive controls were included in reaction steps and all positivities were reproducible. Moreover, amplified products corresponded to those resulting from tumour tissue if available.

In five of eight positive cases, we detected tumour cells only in some samples taken at the same time from different sites (one out of two samples four times and two out of three samples in one patient). This demonstrates the importance of collecting several BM samples for more precise staging and better MRD detection.

Our results confirmed that more than 1/4 of the patients with presumed localized ESFT have minimal BM infiltration. The presence of a low number of cancer cells in BM, detected by sensitive molecular biology techniques including RT-PCR, is not a reason for applying more intensive therapy at the present time (in patients with localized disease), and the clinical significance of minimal BM infiltration at the time of disease diagnosis is unknown. In the future, some new therapeutic protocols might be designed with targeted therapy for patients with proved minimal BM infiltration.

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