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Diagnostic value of the molecular genetic detection of the t(11;22) translocation in Ewing's tumours

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Abstract One consistent feature of the Ewing's tumour family is the presence of a balanced translocation involving band q12 and band q24 of chromosome 22 and chromosome 11. Recent cloning of the chromosome breakpoint regions of t(11;22)(q24;q12) Ewing's sarcoma translocation has revealed that the breakpoints were localized within the Ewing's sarcoma gene (EWS gene) on chromosome 22 and the Fli-1 gene on chromosome 11. Molecular genetic techniques can thus be applied to the detection of the t(11;22) translocation in Ewing's tumours. By reverse transcription and polymerase chain reaction technique (RT-PCR) 11 Ewing's tumour derived cell lines, 12 primary Ewing's tumours, and 11 tumours after treatment were analysed for the occurence of the t(11;22) translocation. Furthermore, blood and bone marrow samples from 5 patients were available for RT-PCR. In 78% of the cell lines and 91% of the primary Ewing's tumours the t(11;22) translocation was detectable by RT-PCR. In bone marrow samples from a Ewing's sarcoma patient presenting in relapse tumour cells were detected by molecular genetic analysis. Our results indicate that molecular genetic detection of the t(11;22) translocation is valuable in the differential diagnosis of small round cell tumours and will provide important information for the staging and prognosis of Ewing's tumour.

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Introduction

The Ewing's tumour family (typical and atypical Ewing's sarcomas and malignant peripheral neuroectodermal tumour, MPNT) shows very little evidence of differentiation on light microscopical examination. These malignancies present a significant differential diagnostic challenge to pathologists. Electron microscopy, immunohistochemical staining for neural antigens such as neuron-specific enolase (NSE), Leu-7, synaptophysin [9, 22, 24, 26, 30] and more recently the detection of MIC-2 gene protein expression have all been included in routine histopathological diagnostic procedures [1, 13, 16]. However, the expression of these antigens is not restricted to this entity [3, 4, 14, 23] and other techniques are necessary to improve diagnostic reliability. One consistent feature of the Ewing's tumour family is the presence of the reciprocal chromosomal t(11;22) (q24;q12) translocation [2, 27, 31, 33]. Recent cloning of the t(11;22) breakpoints has led to the identification of the genes involved in this genetic alteration [35]. Fusion of the human FLI-1 gene on chromosome 11q24 with a gene of unknown function called EWS (Ewing's sarcoma gene) on 22q12 results in a variety of EWS/FLI-1 fusion transcripts which can be detected by molecular genetic analysis [8].

We employed reverse transcriptase polymerase chain reaction (RT-PCR) with oligonucleotide primers derived from EWS and FLI-1 gene sequences to evaluate Ewing's tumour cell lines, primary Ewing's tumours and bone marrow aspirates as well as peripheral blood samples for the occurence of EWS/Fli-1 fusion transcripts.

Materials and methods

The Ewing's tumour cell lines used in this study are listed in Table 1. Cell lines were obtained from the American Type Culture Col-

Table 1 Cell lines (*ES*, Ewing's sarcoma; *MPNT*, malignant peripheral neuroectodermal tumour; *n.d.*, not determined)

Cell line	Diagnosis	Chromosome 22 rearrangements	EWS/FLI-1-Type
A673	MPNT	n.d.	I
STA-ET-1	MPNT	11/22[a]	I
STA-ET-2.1	MPNT	del(22)(q12)[a]	VII
STA-ET-2.2	MPNT	del(22)(q12)[a]	VII
STA-ET-3	MPNT	11/22[a]	I
STA-ET-5	ES	normal karyotyp	–
GG-62	atyp. ES	del(22)(q12)	–
JS-73	ES	n.d.	V
RD-ES	ES	del(22)(q12)[a]	II
RM-82	ES	del(22)(q12)	–
SK-ES-1	ES	11/22[a]	II
SK-N-MC	MPNT	11/22[a]	I
VH-64	ES	11/22	II
WE-68	ES	del(22)(q12)	I

[a] [1]

lection (Rockville, MD) (SK-ES-1, SK-N-MC, RDES). The lines GG-62, JS-73, RM-82, VH-64 and WE-68 were established by culturing primary Ewing's tumour cells using standard methods [32]. Twelve of 14 cell lines have been characterized cytogenetically and details of the cell line A 673 have been published previously [34].

Ewing's tumour specimens obtained from primary biopies or surgical resections after local and/or systemic therapy were classified by a combination of light microscopy, their ultrastructural features, and the results of immunohistochemical investigations [24]. Response to preoperative therapy was evaluated according to Salzer-Kuntschik et al. [25].

Tissue used for RT-PCR were snap frozen in liquid nitrogen and stored at –70°C.

Treatment of patients followed the recommendations of the European Intergroup Cooperative Ewing Sarcoma Study (EICESS).

Total RNA preparation was performed directly from confluent cell cultures or 5 µm thick sections of frozen tumour tissue using the acid-guanidinium-phenol/chloroform method according to standard protocols [5]. From peripheral blood and bone marrow samples total RNA was directly extracted after lysing of red blood cells in 155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM sodium-EDTA pH 7.4 by the acid-guanidinium-phenol/chloroform method. RNA was precipitated by ethanol and stored at –70°C.

Total RNA was converted to cDNA utilizing a first strand cDNA synthesis kit according to the manufacture's protocol (Pharmacia, Biotech). In this procedure either oligo(dT) primer or Fli-1 sequence specific primers were used for generating first strand cDNA in a final reaction mix of 5 µl.

A 1 µl to 4 µl sample of the first strand synthesis reaction was used for amplification in a 50 µl standard PCR reaction employing the primer pair A (5′-CCACTAGTTACCCACCCCAAAC-3′, position 734-755 of the HSEWS-cDNA-sequence, GenBank/EMBL accession number X66899 and 5′-GTGATACAGCTGGCGTTGG CG-3′, position 1024-1044 of the HSHUMFLI-cDNA-sequence, GenBank/EMBL accession number X67001). For bone marrow and peripheral blood samples a second "nested PCR" was added using the internal primer pair B (5′-TCCTACAGCCAAGCTCCA AGTC-3′ and 5′-GAATTGCCACAGCTGGATCTGC-3′). For both PCR steps amplification was achieved by 30 cycles with denaturation at 94°C for 30 s, annealing of the primers at 65°C for 30 s, and extending at 72°C for 45 s. The suitability of the extracted RNA was tested in each case by PCR amplification of a 495 bp fragment of the human β-actin gene with the following primers 5′CATGCCATCCTGCGTCTGGAC 3′, 5′CACGGAGTACTTGC GCTCAGGAGG 3′. Negative controls were included at all steps of the procedure using RNA from unaffected individuals and cytogenetically t(11;22) translocation negative cell lines.

Detection of PCR products were performed by ultrathin native 12% polyacrylamid gelelectrophoresis using a temperature controlled horizontal electrophoresis system (Multiphor, Pharmacia, Biotech). Bands were visualised by silver staining according to standard protocols.

Sequence analysis was performed by solid phase sequencing of single stranded PCR products as previously described [10]. PCR fragments were obtained by amplification with the primer pair A from which one primer was biotinylated. Oligonucleotide primers from primer pair B were used as internal sequencing primers. DNA sequences of the PCR products were determined by dideoxy sequencing using fluorescein-15-dATP as internal label and T7 polymerase (AutoRead Kit, Pharmacia, Biotech). Gelelectrophoresis, data collection and analysis were performed on an automated laser fluorescence sequencer (A.L.F. Pharmacia, Biotech).

Results

RT-PCR revealed EWS/Fli-1 fusion transcripts in 11 (78%) out of 13 cell lines. Among the 11 amplified products, four different sizes of the amplification products were observed representing EWS/Fli-1 fusion transcripts (Fig. 1). They correspond to the previously described type I and type II EWS/Fli-1 fusion transcripts [8]. The sequence of the two other EWS/Fli-1 fusion transcripts of 649 bp (type V) and 820 bp (type VII) revealed in-frame fusion of EWS exon 10 and 9 to Fli-1 exon 5 and 4 respectively (Fig. 4). Table 1 summarizes the RT-PCR results obtained from Ewing's tumour cell lines. Twelve cell lines have been already karyotyped exhibiting rearrangements of the EWS gene. EWS/Fli-1 fusion transcripts could be shown in all cell lines with a cytogenetically demonstrated EWS rearrangement. Two out of

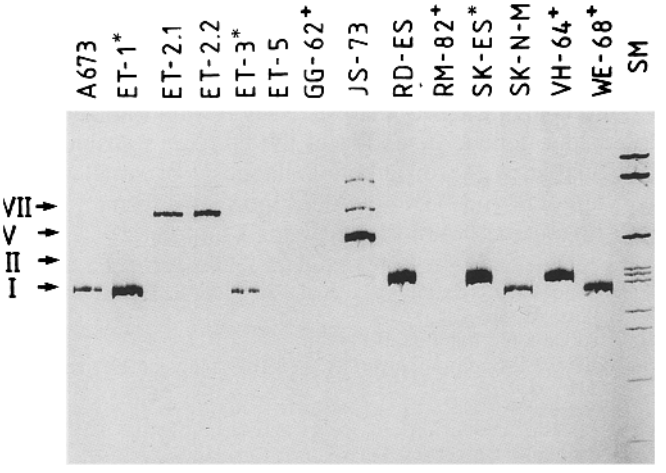

Fig. 1 Reverse transcription and polymerase chain reaction (RT-PCR) of Ewing's cell lines. Amplified PCR products were subjected to a 12% native polyacrylamide gelelectrophoresis (PAGE). Electrophoresis revealed PCR products corresponding to the EWS/Fli-1 fusion transcripts I, II, and VII. Note type V resulted in two additional variant bands which are likely due to different conformations of the PCR product showing different mobilities in native PAGE. When using agarose gelelectrophoresis for separation only one band was obtained (data not shown). SM: size marker (Phi X 174, DNA HaeIII Digest)

Table 2 Pretherapeutic

Patient no.	Diagnosis	EWS/FLI-1-Type
2	ES	I
3	ES	I
4	ES	I
6	ES	III
7	MPNT	I
9*	ES	V
12	ES extraosseous	V
13	ES	II
14	ES	–
26	ES	I
86	ES	VI
132	ES	I

[a] Corresponding tumour to cell line JS-73

Table 3 Posttherapeutic

Patien no.	Diagnosis	Grade of regression	EWS/FLI-1-Type
5	ES	I	–
8	MPNT	III	I
11	ES	I	–
15	ES	I	I
16	ES	IV	II
113	ES	I	–
115	ES	V	I
133[a]	ES	tumour recurrence	I
134	ES	I	I
140	ES	I	–
141	ES	I	–

[a] Tumour recurrence 20 months after initial polychemotherapy

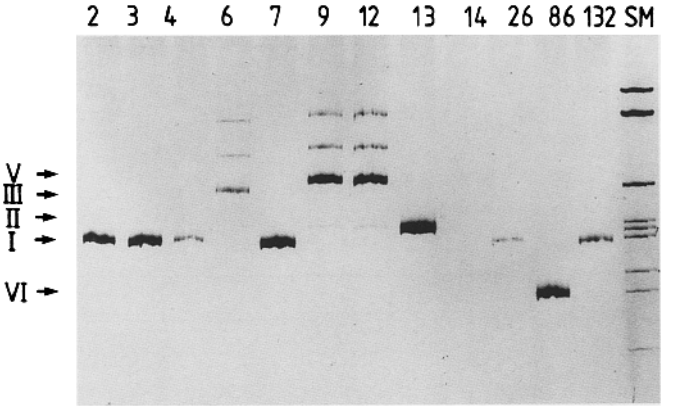

Fig. 2 RT-PCR of primary Ewing's tumour material. PCR products corresponded to the EWS/Fli-1 fusion transcripts type I, II, III, V, and VI when analysed in 12% native PAGE. The two additional variant bands revealed by type V only occur in native PAGE but not in agarose gelectrophoresis (data not shown) and may be likely due to different secondary structures of the PCR product as discussed for type V in Fig. 1

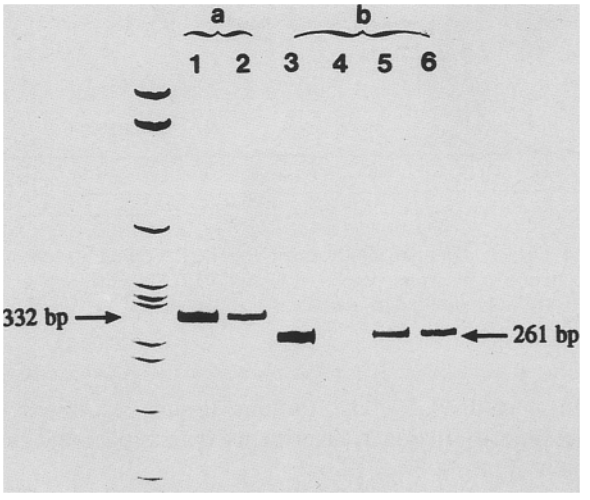

Fig. 3 Native 12% PAGE. (**a**) First round of PCR resulting in a 332 bp fragment corresponding to EWS/FLI-1 fusion transcript type I. Lane 1: positive control; lane 2: tumour tissue (no. 133; table 3). (**b**) Nested PCR revealing a 261 bp product. Lane 3: tumour tissue; lane 4: peripheral blood sample; lane 5 and 6: bone marrow samples from pelvic crest and sternum

the six cell lines with the chromosomal aberration del(22)(q12) were negative for EWS/Fli-1 fusion transcripts. ET-2.1 was cultured from a primary Ewing's sarcoma and ET-2.2 from the bone metastasis of this tumour. Both samples revealed EWS/Fli-1 fusion transcripts type VII. Cell line A673 was published first described as being derived from a primary rhabdomyosarcoma. By RT-PCR analysis a type I EWS/Fli-1 fusion transcript was found.

The feasibility of our approach for tumour tissue was tested analysing 12 samples from primary biopsies and 11 samples derived from surgical resections after therapy. RT-PCR results are listed in Table 2 for biopsy material obtained at the initial diagnosis and in Table 3 for the resection specimen. Of the primary Ewing's tumours 91% revealed EWS/Fli-1 fusion transcripts of types I, II, III, and V as well as a PCR fragment of 205 bp (type VI) comprising EWS exon 7 to Fli-1 exon 8 in frame fusion (Fig. 2 and 4). Among the 11 surgical tumour resections after chemotherapy and/or radiation therapy typical EWS/Fli-1 fusion transcripts was demonstrated in six cases. When compared with the grade of tumour regression in those tumours with poor response to therapy (no. 8, 16, 115) EWS/Fli-1 fusion transcripts were detected. In 2/7 Ewing's tumours which have been morphologically classified as completely devitalized (grade 1) EWS/Fli-1 fusion transcripts were detected by RT-PCR.

Bone marrow aspirates from 5 patients with known EWS/Fli-1 fusion transcripts in their primary tumour and peripheral blood samples were available for RT-PCR. For this analysis RT-PCR was modified by addition of a second round of PCR using internal primers (nested PCR) to increase the sensitivity of the method. An EWS/Fli-1 fusion transcript type I was found in the bone marrow aspirates of one patient with disseminated Ewing's sarcoma presenting in relapse, where cytological examination failed to detect tumour cells. The corresponding peripheral blood sample was negative in RT-PCR and in subse-

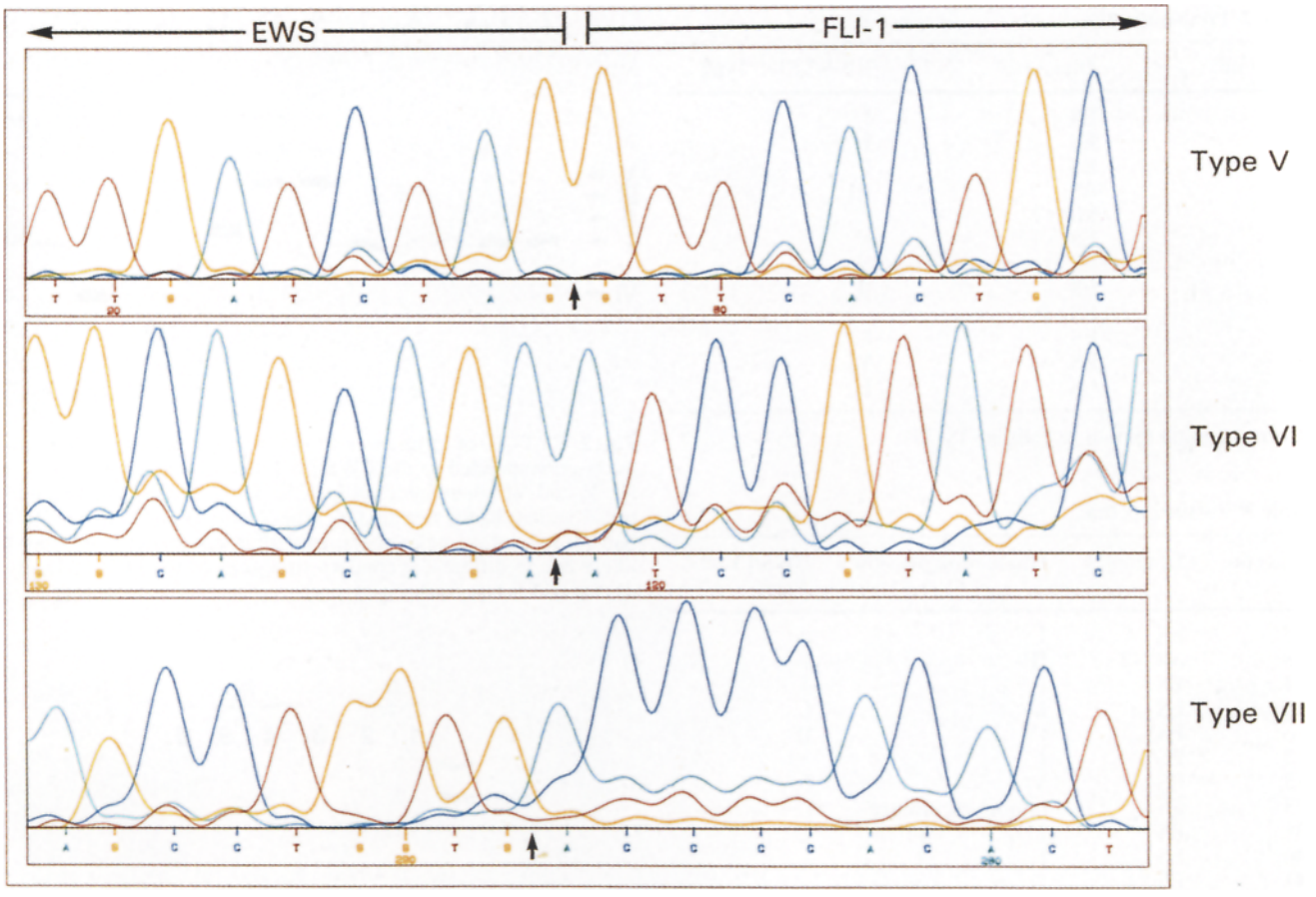

Fig. 4 Direct sequence analysis of the PCR fragments obtained from fusion transcripts type V, VI, and VII. The different breakpoints are indicated by an *arrow*

quent nested PCR. The tumour tissues exhibited the same type of fusion transcript as detected in the bone marrow (Fig. 3).

The specificity of RT-PCR based detection of EWS/Fli-1 fusion transcripts for Ewing's tumours was confirmed by examination of further 25 tumours, comprising neuroblastoma, fibrosarcoma, rhabdomyosarcoma, chondrosarcoma and osteosarcoma. In none of these tumours EWS/Fli-1 fusion transcript was found.

Discussion

Since chromosomal t(11;22) translocation has been found to be a constant feature in tumours of the Ewing's family cytogenetic analysis has been extremely useful in diagnostic work-up of small round cell tumours [15, 31]. However, cytogenetic analysis requires fresh tissue from solid tumours which often fail to grow in culture. Furthermore, the involvement of cytogenetic findings in routine diagnosis is hampered because it often takes weeks before results are available and only few laboratories are expert in performing this procedure. Cloning of the breakpoint region of the t(11;22) translocation [35] and subsequent identification and characterisation of the involved genes [8] has led to the applicability of molecular genetic techniques for the detection of this genetic abnormality [12, 28]. The validity of molecular genetic detection of the t(11;22) translocation employing RT-PCR has been demonstrated by analysing cell lines derived from Ewing's tumours and primary tumour tissue. Cytogenetic identification of the t(11;22) translocation in cell lines correlated with the detection of EWS/Fli-1 fusion transcripts by RT-PCR. The detection of EWS/FLI-1 fusion transcripts in cell lines which exhibit cytogenetically del(22)(q12) chromosomal aberrations led to the suggestion that the majority of tumour cells contain the deletion of chromosome 22, del(22)(q12), whereas only in a few tumour cells has the complete t(11;22) translocation occurred. The high sensitivity of RT-PCR allows us to identify t(11;22) translocation specific EWS/Fli-1 fusion transcripts even in a low number of tumour cells. Interestingly the cell line A673 derived from a tumour which was first diagnosed as an rhabdomyosarcoma bears an EWS/Fli-1 fusion transcript type I. A finding which agrees with recently published reports suggesting that the primary tumour has to be reclassified as a MPNT [28].

Examination of primary tumour tissue revealed EWS/Fli-1 fusion transcripts in 91% of cases. Types of fusion transcripts detected in primary tumours and the corresponding cell lines were identical as shown in case 9 (Table 2) and JS-73 (Table 1) respectively. Cell lines de-

rived from a primary Ewing's tumour and the corresponding lung metastasis exhibited the same EWS/Fli-1 transcript. These findings demonstrate the validity of RT-PCR analysis for the primary diagnosis of small round cell tumours and its usefulness in detecting metastatic sites. Furthermore, analysis of surgical tumour resection after therapy demonstrated that the molecular genetic approach can be used as an adjunct to surgical pathology in evaluating the grade of tumour regression and response to chemotherapy. Cell lines and primary tumours that lack detectable EWS/Fli-1 fusion transcripts may exhibt a genetic alteration different from the t(11;22) translocation [11]. We believe it is likely that either a deletion of 22, del(22)(q12), or a translocation, t(21;22), recently described for a minority of Ewing's tumours, has occurred in our negative cases [29].

In our study six different EWS/Fli-1 fusion transcripts were found which varied the size of the resulting PCR products. EWS/Fli-1 fusion transcript type I was detected in 14 (52%) cases predominantly. The specificity of these fusion transcripts was confirmed by direct sequence analysis revealing complete homology with the known human EWS and Fli-1 sequences. In all cases the fusion was in frame and the resulting putative chimeric proteins differed from the EWS protein by the substitution of the putative RNA-binding domain and by the DNA-binding domain of the human Fli-1 gene when compared with the recently published sequence data [20, 36]. The observed heterogenity is thought to arise from variation in the t(11;22) genomic breakpoints. Alternative mRNA splicing may be a second mechanism causing heterogenity of t(11;22) products as observed for the EWS/Fli-1 fusion transcript type IV, co-expressing two distinct EWS/Fli-1 chimeras [18]. A type IV EWS/Fli-1 fusion transcript was not found in the cases examined in the course of our study. As is known from leukaemia studies translocations resulting in distinct transforming products can cause a similiar if not identical leukaemic phenotype [6, 7, 21]. May et al. [19] have shown that EWS/Fli-1 fusion proteins can act as strong transcriptional activators and have a higher cell transforming activity than Fli-1 protein alone. Furthermore, the N-terminal portion of EWS (NTD-EWS) has been shown to contain a potent transcription activation domain and appears to be required since it is systematically observed in the chimeric proteins containing either Fli-1 or erg [36]. Thus the NTD-EWS and the ets domain of either Fli-1 or erg are suggested to be constant features of the hybrid protein and may both be required for its oncogenic potential. Although, there was no evident association between tumour phenotype and the specific rearrangements found in the course of our study further long-term studies regarding individual clinical outcome of the Ewing's tumour patients will allow evaluation of the possibility that various EWS/Fli-1 fusion transcripts are associated with different biological behaviour.

In order to increase the sensitivity of RT-PCR for the detection of tumour cells contaminating peripheral blood and bone marrow samples a nested PCR was added following the first round of PCR. Bone marrow and peripheral blood samples were available from five patients with a known EWS/Fli-1 fusion transcript in their primary tumour. With nested PCR residual tumour cells were detected in two bone marrow samples obtained from distinct locations from a patient who was presented in relapse with multifocal bone disease. Cytological examination of the respective bone marrow samples was negative. As in leukaemia patients with reciprocal t(9;22) translocation, RT-PCR and subsequent nested PCR allows us to detect extremely small numbers of cells carrying the translocation [17] and has already become a basic part of the diagnostic workup of the disease. The application of a two-step RT-PCR assay provides a powerful tool to examine Ewing's tumour patients for occult micrometastatic spread at the time of diagnosis and during systemic therapy and follow-up. Furthermore, evaluation of bone marrow and peripheral blood stem cell grafts for contamination of residual tumour cells employing two-step RT-PCR prior to autologous transplantation may become an important step in the transplantation procedure.

Molecular genetic detection of the t(11;22) translocation opens a new modality for accurate histological diagnosis of small round cell tumours. Since Ewing's tumour patients require therapeutic stratification based on appropriate staging the evaluation of peripheral blood and bone marrow samples and of bone marrow and peripheral stem cell grafts prior to transplantation may be useful in customising risk adapted therapy, including myeloablative therapy and stem cell grafting.

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