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# Detection of EWS chimeric transcripts by nested RT-PCR to allow reinfusion of uncontaminated peripheral blood stem cells in high-risk Ewing's tumor in childhood

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**ABSTRACT**

***Background and Objectives.*** **Ewing's tumors (ET) are primary malignancies of bone and soft tissues characterized in at least 96% of cases by specific fusion transcripts originating from recurrent chromosomal translocations. Clinical protocols for high-risk metastatic ETs include high-dose radiation/chemotherapy followed by autologous peripheral blood stem cell (PBSC) reinfusion. We used nested reverse transcriptase polymerase chain reaction (RT-PCR) to search for the presence of ET-specific transcripts in PBSC collections from patients with high-risk ET in order to collect harvests free from neoplastic cells but still sufficient to obtain early stable engraftment.**

***Design and Methods.*** **Thirty-seven harvest samples from 15 ET patients treated with mobilizing chemotherapy were analyzed. Nested RT-PCR was performed to detect ET-specific transcripts in RNA extracted from the PBSC collections.**

***Results.*** **A total of 30 harvests was performed. On average, 2 harvests (range 1-4) were sufficient to collect the minimum required number of mononuclear cells ($2.5\times10^6$/kg). Nested RT-PCR revealed neoplastic cells in 4/30 (13%) harvests, which were derived from 3/15 patients (20%). After further cytoreductive/mobilizing chemotherapy, a total of 7 additional harvests taken from these 3 patients were all free from neoplastic cells.**

***Interpretation and Conclusions.*** **PBSC collections from ET patients undergoing autologous stem cell transplantation are at risk of neoplastic contamination. Additional harvests following a further cycle of cytoreductive/mobilizing therapy may be sufficient to obtain non-contaminated material for reinfusion.**
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Key words: Ewing's tumor, neoplastic contamination, PBSC, ets/EWS chimeric transcripts, nested RT-PCR

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Ewing's tumors (ET) are primitive malignancies of bone and soft tissues. At least 96% of ET are characterized by specific *ets*/EWS chimeric transcripts,[1] which derive from rearrangements of the EWS gene located on chromosome 22q12 with one of the *ets* family of oncogenes. The *ets* oncogenes involved in ET-specific chromosomal rearrangements are FLI1, ERG, ETV1 and E1AF, which are located on chromosomes 11q24, 21q22, 7q22 and 17q22 respectively.[2-6] By far the most frequent fusion transcript is EWS/FLI1, which is present in approximately 90% of ET, while an alternative transcript, EWS/ERG, is found in 6% of cases.[1] The nested reverse transcription polymerase chain reaction (RT-PCR) technique, which is the most sensitive method for detecting the presence of these transcripts, has been used to evaluate the presence of ET cells in peripheral blood and bone marrow at diagnosis and to monitor minimal residual disease during and after therapy.[7-9]

About 65% of ET are considered to be at high risk because of the presence of a large primary mass or metastasis at the time of diagnosis. Clinical protocols proposed for treatment of high-risk ET include high-dose radiation and chemotherapy followed by autologous peripheral blood stem cell (PBSC) reinfusion.[10,11] Neoplastic cells have been detected in PBSC harvests from patients with several solid and hematologic malignancies submitted to a similar therapeutic approach.[12]

In the present study, we searched for the presence of *ets*/EWS fusion transcripts by nested RT-PCR in PBSC harvests taken from high-risk ET patients in order to detect minimal contamination by neoplastic cells in the samples assigned to autologous reinfusion after myeloablative radiation and chemotherapy.

## Design and Methods

### *PBSC harvests*

Thirty PBSC harvests from 15 high-risk ET patients enrolled in the Italian national pilot study SE'93-AR were studied. Of these 15 patients (primary mass: 7 in the pelvis, 4 in the ribs, 4 in the limbs) 12 had

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metastatic disease at diagnosis (9 in the lungs, 3 in the bones) and 3 had a localized pelvic mass greater than 200 mL. In this pilot study, PBSC were collected at an early phase of the induction-mobilization chemotherapeutic step. Induction consisted of 2 courses of HyperVAC (vincristine, cyclophosphamide, adriamycin), alternated with 2 courses of CE (cyclophosphamide, etoposide) in order to reduce the primary and/or metastatic disease and to mobilize hematopoietic stem cells into the peripheral blood. Each cycle of CE was supported by 250 μg/m²/day of granulocyte-colony stimulating factor (G-CSF) (filgrastim) administered from day 5 after the end of chemotherapy up to PBSC collection. A blood sample to determine $CD34^+$ number was obtained daily after the nadir count of polymorphonuclear cells. PBSC collection started when the number of circulating $CD34^+$ was >20/μL independently of WBC and platelet count. The apheresis continued until a minimum number of $2.5\times10^6$/kg of $CD34^+$ cells were collected by means of the COBE instrument and the SPECTRA method applying specific gravities of 1,085-1,092 and 1,050-1,060 for polymorphonuclear and mononuclear cells respectively.[13] In cases in which at least one collection was found to be contaminated, additional leukaphereses were performed after a further cycle of hyperVAC followed by CE.

### *RNA extraction and RT-PCR analysis*

All determinations were performed in the same institution (Bologna) using samples from several hospitals with strict standardization according to the following protocol. In all cases RNA extraction was performed within 24h of harvesting. Samples were centrifuged for 5 min at 1,000g, and cells were lysed in RNAfast (Biotecx, CA, USA) solution. Total RNA was obtained following the standard protocol recommended for this product. From 2 to 4 μg of RNA were reverse transcribed by a 1st strand cDNA synthesis kit utilizing random hexamers (Boehringer Mannheim, Germany). To avoid false negativity due to extracted RNA degradation, we examined the constitutive $\beta_2$-microglobulin transcript using the recommended primers.[14] cDNA was then subjected to a specific amplification for chimeric transcripts EWS/FLI-1 and EWS/ERG. The PCR reaction contained $MgCl_2$ 1.5 mM, dNTPs 200 μM, 7.5 pmol/50 μL of each primer and the appropriate Taq polymerase buffer provided by the manufacturer (Boehringer Mannheim, Germany). After an initial denaturing step of 10 min at 94°C, 2 U of Taq polymerase were added. In accordance with Pfleiderer *et al.*,[8] first-round PCR was performed with primers 11.3 (5'-ACT CCC CTT GGT CCC CTC C) and 22.3 (5'-TCC TAC AGC CAA GCT CCA AGT C) for EWS/FLI1, and with 22.3 and ERG.3 (5'-ACT CCC CGT TGG TGC CTT CC) for EWS/ERG at the following conditions: 30 cycles at 94°C for 30 sec, followed by 65°C for 1 min and 72°C for 1 min. Two μL of first-round PCR product were subjected to nested PCR. Nested PCR reaction was done using primers 11.4 (5'-CAG GTG ATA CAG CTG GCG) or ERG.4 (5'-CAG GTG ATG CAG CTG GAG) and 22.4 (5'-CCA ACA GAG CAG CAG CTA C). All amplification products were fractionated through a 2% agarose gel together with a molecular weight marker (Marker VI, Boehringer Mannheim) and ethidium bromide stained. All steps included positive (LAP35 for EWS/FLI1 and characterized tumor samples for EWS/ERG)[15,14] and negative (no template) controls. In order to minimize the risk of contamination by PCR product carry over, extraction, amplification and electrophoresis were performed in separate rooms using specifically designated sets of micropipettes with disposable filter tips. The sensitivity of the method was evaluated in serial dilution (ranging from 1 neoplastic cell in $1\times10^3$ mononuclear cells to 1 in $1\times10^8$) in whole blood of the LAP35 cell line harboring the t(11;22)(q24;q12).[15]

When indicated amplification products were purified and sequenced in an Applied Biosystem 377-18 DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) using a BigDye terminator kit (Perkin-Elmer).

## Results

### *Evaluation of nested RT-PCR sensitivity*

Before analyzing the PBSC samples of patients, we assessed the sensitivity of the method on progressive dilutions of LAP 35, an ET-derived cell line characterized by t(11;22)(q24;q12) in whole peripheral blood. Dilutions were 1 neoplastic cell in $1\times10^3$, $1\times10^4$, $1\times10^5$, $5\times10^5$, $1\times10^6$, $1\times10^7$, $1\times10^8$ whole blood mononuclear cells. An amplification band of approximately 346 base pairs (bp), corresponding to the EWS/FLI1 type 2 fusion transcript present in LAP 35 cells, was detected in up to a dilution of 1 tumor cell in $1\times10^5$ normal mononuclear cell after nested RT-PCR (Figure 1).

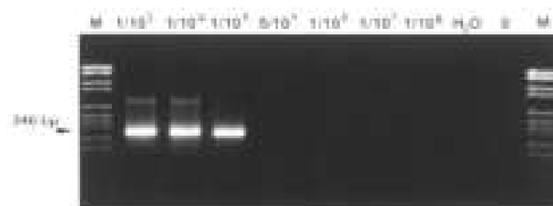

**Figure 1. Results of the sensitivity test of nested RT-PCR analysis. M: Boehringer Mannheim Molecular Weight Marker VI. Serial dilutions of the LAP35 cell line harboring the t(11;22)(q24;q12) were evaluated. It is possible to detect an amplification band in up to a dilution of 1 tumor cell in $1\times10^5$ normal peripheral blood mononuclear cells.**

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**Table 1. Results of nested RT-PCR analysis of each patient.**

| Patient | Harvests | | Transcription type | |
|---|---|---|---|---|
| | *early pos./total* | *additional pos./total* | *detected in PBSC* | *detected in primary tumor* |
| 1 | 0/2 | | | |
| 2 | 0/2 | | | |
| 3 | 0/2 | | | |
| 4 | 0/2 | | | |
| 5 | 0/2 | | | |
| 6 | 0/1 | | | t(11;22) type 1 |
| 7 | 1/3 | 0/2 | t(11;22) type 1 | t(11;22) type 1 |
| 8 | 1/2 | 0/4 | t(11;22) type 8 | |
| 9 | 2/2 | 0/1 | t(11;22) type 9 | |
| 10 | 0/4 | | | t(11;22) type 2 |
| 11 | 0/2 | | | |
| 12 | 0/1 | | | |
| 13 | 0/1 | | | |
| 14 | 0/2 | | | |
| 15 | 0/1 | | | |
| Total | 4/30 | 0/7 | 3/15 | |

### *Patient samples*

Of the first 30 harvests analyzed, 4 (13%) turned out to be positive after nested RT-PCR for the presence of the t(11;22)(q22;q12) derived EWS/FLI1 fusion transcript. The 4 positive harvests came from 3/15 (20%) patients (Table 1). On the basis of the molecular weight of the amplification bands obtained, the detected chimeric transcripts were defined as EWS/FLI1 type 1 (279 bp), type 8 (154 bp) and type 9 (105 bp) in patients #7, 8 and 9, respectively. No tested sample was positive for the EWS/ERG fusion transcript characteristic of t(21;22) (q24;q12). As shown in Table 1 data on the primary tumor were available only for a small number of patients. When possible the specificity of the transcript type was confirmed by comparing it with the one detected in the primary tumor (sample #7). In the other two cases the specificity of the nested RT-PCR assay was confirmed by sequencing the amplification product.

As can be seen from Table 1, a total of 7 additional harvests was performed after a further cycle of cytoreductive and mobilizing therapy in the 3 patients who had had at least one contaminated early collection. Since none of the previously identified EWS/FLI1 fusion transcripts was detected by nested RT-PCR analysis, these collections were considered to be free from neoplastic cells. The additional harvests permitted reinfusion with only non-contaminated material.

Of the 15 patients considered in the study, 7 had local recurrence of disease after a median time of 7 (min 2, max 24) months from PBSC transplantation, 6 are alive and free from progression of disease after a median time of 21 (min 6, max 28) months from PBSC transplantation. Two patients were lost to follow-up. None of our patients had bone marrow and/or disseminated recurrence of disease after autologous PBSC transplantation.

## Discussion

One possible pitfall of autologous PBSC transplantation is the risk of reinfusion after high-dose radiation and chemotherapy of contaminated cell harvests. Neoplastic contamination of PBSC harvests has been observed in several malignancies, including neuroblastoma,[16,17] leukemias and lymphomas,[18-20] melanoma[21] and breast cancer.[22] In some tumors, such as breast cancer and melanoma, such contamination has no observable effect on clinical outcome,[21,22] but in others, such as neuroblastoma and myeloma, it has been shown to be potentially clonogenic or to contribute to relapse.[23,24]

In this study, we used nested RT-PCR to detect contaminated PBSC collections in patients with high-risk ET. Our results indicate that this technique can detect up to one tumor cell in $1\times10^5$ mononuclear cells in whole peripheral blood, whereas other authors[7,8] reported a sensitivity of one tumor cell in $1\times10^6$ normal cells. This discrepancy might depend on the particular cell line (LAP35) we utilized for the serial dilution experiments, which we suspect might express different levels of fusion transcript with respect to the cell lines employed elsewhere.

In one of the two previous studies utilizing nested RT-PCR to evaluate the purity of PBSC collections from ET patients, neoplastic cells were detected in 2/18 leukaphereses from an unspecified number of patients,[7] while in the other study no contamination was found in any of the harvests of 5 patients.[25] In our larger series of patients, 4/30 (13%) of the original collections turned out to be contaminated, and the risk to the patients of receiving at least one contaminated collected product would have been considerable (3/15, 20%). In the absence of any information regarding the clonogenicity of the ET cells present in PBSC or the clinical consequences of the reinfusion of minimally contaminated material, it seems sensible, when possible, to use only nested RT-PCR negative collections for reinfusion. The observation that none of the patients grafted showed bone marrow relapse and that the disease in the seven patients who did relapse after stem cell transplantation reoccurred at the sites at which it was found at diagnosis encourages us to think that an additional cycle of therapy prior to repeated harvesting could be a suitable approach for patients affected by high-risk Ewing's sarcoma with contaminated samples. Probably this approach would be sufficient to avoid metastatic dissemination caused by neoplastic cells contaminating the infusions.

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*LM designed the study, participated in the analysis of molecular data and wrote the manuscript, APe participated in the design of the study, analysis of clinical data and revised the manuscript during its writing, DT contributed to data analysis and revising the manuscript, MV contributed to molecular data analysis and manuscript writing, APr performed leukaphereses, collected clinical data and contributed to writing the paper, GP contributed to the review of the paper, MD contributed to the design of the study and finally revised the paper.*

*The order takes into account the time, work and scientific contribution given by all authors. GP and MD, as senior authors, are cited last.*

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### Disclosures

*Conflict of interest: none.*

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### Manuscript processing

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