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Clinical and molecular follow-up by amplification of the CDR-III IgH region in multiple myeloma patients after autologous transplantation of hematopoietic CD34+ stem cells

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ABSTRACT

Background and Objective. **Autologous blood stem cell transplantation (ABSCT) using chemotherapy-induced mobilization of peripheral blood stem cells (PBSC) is being increasingly used in the treatment of multiple myeloma (MM). We report the clinical and molecular follow-up of 10 MM patients who underwent autologous stem cell transplantation with peripheral blood selected CD34+ cells, as support therapy following a myeloablative conditioning regimen.**

Design and Methods. **The CDR-III coding region of the IgH gene was studied by a) consensus PCR applied to 8 MM patients, or b) by direct sequencing of PCR product generated by family-specific primers in the remaining two patients (who became immunofixation analysis (IF) negative). In this case, two patient-specific primers were generated, thus obtaining a high PCR assay sensitivity and specificity (ASO PCR).**

Results. **Seven patients are alive: 4 of them have serum M protein assessable by IF, while 1 was not a secretor and 2 converted from serum IF positivity to negativity 6 and 12 months after ABSCT. Three patients died: 1 from disease progression and 2 from infective complications during clinical remission. The molecular analysis during the follow-up showed that the bone marrow samples from the two patients who obtained IF negativity were persistently PCR positive for the presence of rearranged CDR-III region. Moreover, despite the remarkable reduction of myeloma burden, a minimal level of residual myeloma cells was still detectable by molecular analysis.**

Interpretation and Conclusions. **These results confirm that although positive selection of CD34+ cells markedly reduces the contamination of myeloma cells from apheresis products by up to 3 log, and provides a cell suspension capable of restoring normal hematopoiesis after ablative conditioning regimen, it does not abrogate myeloma cell contamination in most of the apheresis products.**

Key words: minimal residual disease, multiple myeloma, CD34+ cells, ABMT

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Multiple myeloma (MM) is a B-cell-derived neoplastic disease generally associated with the expansion of mature plasma cells, monoclonal Ig production, and multiple osteolytic lesions.[1] Because of the limitations of conventional therapeutic approaches, several regimens involving myeloablative radiochemotherapy followed by the reinfusion of autologous stem cells have been employed.[2-7] The use of autologous stem cells could reduce the morbidity and mortality associated with high-dose therapy-related procedures.[8,9] Thus, many investigators have stressed the option of using blood as an alternative source of autologous hematopoietic stem cells for autografting.[5,9]

Autologous blood stem cell transplantation (ABSCT) using chemotherapy mobilized cells (PBSC) is being increasingly used in the treatment of MM.[9-11] One of the major arguments in favor of ABSCT is the possibility that PBSC harvests may be less contaminated by myeloma cells than bone marrow mononuclear cells (BMMNC).[10,11] However, tumor-related B cells bearing the same idiotypic determinants as neoplastic plasma cells have been identified in the blood of MM patients and they have been shown to belong to the neoplastic stem cell compartment.[12]

Polymerase chain reaction (PCR) has been used for monitoring minimal residual disease (MRD) in MM by amplification of the CDR-III region.[13] Clonal CDR-III region products can be generated by amplification in all instances of MM, thereby providing a specific diagnostic marker for each B cell clone. In fact, the variable regions of immunoglobulin heavy chains (IgH) are encoded by a series of discontinuous germline gene segments, called variable (VH), diversity (D) and joining (JH) regions, which undergo somatic rearrangement at an early stage of B lymphocyte development.[14,15] When a pluripotent hematopoietic stem cell differentiates into the B-cell lineage, the first detectable genetic event is an IgH gene rearrangement in the VH, D and JH regions that consists of two successive somatic recombinations, namely D-JH and VH-D-JH.[16] This region can be considered an identification sequence for members of the same B-cell clone. Rearrangement of the IgH locus in MM presents the

same characteristics and sequence as in normal B-cells.[14,17,18] B-cell lineage hematopoietic malignancies are usually monoclonal and contain one or, more often, two rearranged alleles.[19-21]

It is now well established that CD34+ selected ABSCT leads to stable long term engraftment. In contrast, less information is present in literature regarding the benefit on the response to transplantation, duration of remission and on the level of neoplastic cells in the PB of MM patients after mobilization protocols. Furthermore, tumor cell contamination of patient's bone marrow samples during post-transplant follow-up has been little investigated. We previously reported the analysis of circulating tumor cells by amplification of the CDR-III region in the PBSC of 10 MM patients who underwent ABSCT using CD34+ cells.[9] We now report the clinical outcome and molecular follow-up of the same group of MM patients after ABSCT using CD34+ cells.

Design and Methods

Clinical study

Ten patients with MM diagnosed according to the standard criteria were included in the study (Table 1a). The therapeutic protocol employed was approved by the University hospital ethical committee, and each patient gave written informed consent. Patients were treated with high-dose cyclophosphamide (Cy) (7 g/m^2) as described elsewhere,[9] followed by administration of G-CSF (filgrastim, neupogen, Dompè Biotec, Milan, Italy) at a dose of 5 mg/kg/day s.c. starting on day 2 after chemotherapy and continuing until the completion of PBSC collection. Leukapheresis were performed using a Baxter CS 3000 plus blood cell separator using the modified procedure number 1 program as reported.[9] Circulating hematopoietic CD34+ cells were highly enriched by avidin-biotin immunoabsorption, as previously described,[9] and were cryopreserved prior to use for reconstitution of bone marrow function after myeloablative therapy, immediately following positive selection of CD34+ cells.

CD34+ cells were reinfused on day 0 following the previously reported conditioning regimen.[9] Two patients (#9 and 10) were submitted to a second ABSCT, three months after the first: the conditioning regimen consisted of busulphan (12 mg/kg) (day –5 to –3) and melphalan (120 mg/m^2) (day –2) and was followed by re-infusion of CD34+ cells (day 0). G-CSF at 5 mg/kg/day s.c. was started on day +1 and administered until the granulocyte count had reached >0.5$\times$10^9/L for 3 consecutive days. All patients received a single-donor platelet transfusion if their platelet count was <20$\times$10^9/L and a red blood cell (RBC) transfusion if their hemoglobin level was < 8 g/dL. Patients who achieved a clinical complete remission (CR) or partial remission after transplantation received interferon-α (IFN-α) s.c. (3$\times$10^6 IU/m^2 three times a week), beginning at the time of full hematologic recovery and continuing until there was evidence of disease progression.

Preparation of PB and BM specimens

Preparation of PB and BM specimens for DNA and RNA analyses was performed as described elsewhere.[22] In all patients, the study was performed at diagnosis on bone marrow samples and on aphereses before and after CD34+ selection, and every 3 months during the follow-up. Samples suitable for RNA extraction were collected as reported previously.[23] Serologic CR was defined by the disappearance of the M component at immunofixation analysis, and the resolution of bone marrow plasma cell infiltration to below 3%. Hematopoietic progenitor cell assay, cell phenotype analysis, intracytoplasmic Ig (clg), bromodeoxyuridine (BRDU) staining was performed as reported.[9] Positive selection of CD34+ cells was performed as described elsewhere.[9]

Analysis of minimal residual disease (MRD) by DNA amplification of the CDR-III region of the IgH gene with consensus primers and patients' specific primers

MRD with consensus primers

MRD was analyzed by IgH gene amplification on aphereses as previously described.[9] Briefly, high molecular weight DNA was extracted from bone marrow taken at diagnosis and from leukapheresis collections obtained before and after positive selection of CD34+ cells by proteinase-K digestion followed by phenol chloroform extraction. Amplification was performed essentially as described.[9,24] The sensitivity of our method of detecting the IgH gene rearrangement has been previously assessed.[9]

MRD with patients' specific primers (ASO PCR)

VDJ sequence. VDJ gene rearrangement amplification was performed with a panel of VH family specific primers, together with a JH consensus primer. Seven amplifications were performed for each patient in order to determine the VH segment involved in the myeloma VDJ gene rearrangement. The reaction mixture (50 µL) contained 200 mmol/L dNTPs, 1 X PCR buffer (10 mM βME, 6.7 mM EDTA pH 8, 67 mM Tris pH 8.8, 170 mg/mL BSA), 7.7 mM $MgCl_2$, 50 pmol of each primer, 2% DMSO and 0.3 U Taq DNA polymerase (Boehringer Mannheim, Malta-Italy). Thirty cycles of amplification were performed, consisting of denaturation at 95°C for 30 sec, annealing at 61°C for 40 sec and extension at 72°C for 50 sec, followed by a 7 min final extension at 72°C. A 15 µL aliquot was analyzed by ethidium bromide staining 3% agarose gel: a single discrete 300 bp band was obtained for each patient. A 30 µL aliquot of amplification product, corresponding to the VH family-specific gene rearrangement, was loaded on a 1.25% low melt preparative grade agarose gel (BioRad, Segrate, Italy). The ~300 bp band was excised from the gel and purified with a Gel Nebulizer Micropure™ Separator (Amicon, Italy), according to the manu-

Table 1a. Patients' characteristics.

Characteristics	*no. of patients*
Median age in years (range)	51.5
Sex (M/F)	5/5
Tumor stage*	
I	4
II	3
III	3
M component	
IgG	6
IgA	3
BJ	0
non secretory	1
Light chain	
λ	3
κ	6
non secretory	1
Renal insufficiency	
yes	/
no	10
Previous therapy	
Alkylating agents	2
VAD or VAD-like therapy	3
VAD + alkylating agents	4
α-IFN alone	/
radiotherapy alone	1

**according to Durie-Salmon classification.*

facturer's instructions. An aliquot of purified DNA was directly sequenced with the family-specific VH primer, using the Thermo Sequenase DNA cycle-sequencing kit (Amersham Italia, Italy), according to the manufacturer's instructions. Sequence analysis was performed using PC-GENE software (IntelliGenetics). Patient-specific primers were designed from the sequence information of the CDR-II and CDR-III regions in order to generate clonal patient-specific amplification (Table 1b).

Molecular follow-up of MRD with patients' specific primers. Follow-up studies for the detection of MRD were performed on bone marrow specimens, taken at various times after BMT. The minimum follow-up period for all patients was 3 months, the maximum 48 months. Clonally expanded B-cells were detected by amplifying 1 µg of DNA, using the patient's specific CDR-II and CDR-III primers. Fifty cycles of amplification were performed, consisting of denaturation at 96°C for 30 sec, annealing at the best tested temperature for 30 sec and extension at 72°C for 40 sec, followed by a 7 min. final extension at 72°C. The reaction mixture (50 µL) contained 200 mmol/L dNTPs, 1X PCR buffer (500 mM KCl, 100 mM Tris, pH 8.3), 2.5 mM $MgCl_2$, 50 pmol of each patient-specific primer and 1 U of AmpliTaq Gold™ (Perkin Elmer, Milan, Italy). A 15 µL aliquot was analyzed on agarose gel as described above. A 150 bp ca. band was obtained in each patient analyzed. Sensitivity of each set of primers was checked on serial dilutions of patient's DNA from initial marrow samples in an appropriate amount of DNA from normal peripheral blood. Using this CDR-III ASO PCR assay we were able to increase the sensitivity of detection of MM cells in 10^{-5} to 10^{-6} dilutions.[9]

Results

Clinical data and mobilization of tumor cells and hematopoietic progenitor cells

The clinical characteristics of the patients are reported in Table 1a. All patients received one or more lines of treatment before high dose Cy. None of them was in CR at the time of the study. Of the 10 patients, 2 had been treated with a median of 6 cycles of an alkylating agent-containing regimen (mostly melphalan and prednisone), 3 patients had received 4 cycles of VAD therapy (vincristine, doxorubicin, and dexamethasone), and 4 individuals had received both. One patient received radiotherapy alone (Table 1a). The circulating plasma cells levels were recorded before initiation of PBSC mobilization and have been already reported.[9] Kinetic analysis showed a pattern of tumor cell mobilization similar to that of normal hematopoietic progenitor cells, with a maximum peak falling within the optimal time period for the collection of PBSCs.

Ex vivo purging of tumor cells by positive selection of CD34+ cells

Contamination of neoplastic plasma cells and B cells in 8 patients has been reported.[9]

All 10 MM patients had their circulating CD34+ cells purified by the Ceprate SC concentrator. After positive selection a reduction of 99.7±0.2% of plasma cells was documented by microscope immuno-

Table 1b. Sequences of the clonal CDR-III region of the IgH gene of the two MM patients undergoing molecular follow-up with patient-specific primers. VH-family = family specific VH segment used in the V-D-J rearrangement. Primers (sense and antisense) are given as 5'-3' sequences. Annealing temperatures are reported.

No.	*Name*	*VH family*	*Primer sense (5'-3')*	*CDR-III sequences (5'-3')*	*Primer antisense (5'-3')*	*Annealing temp.*	*Size of PCR product*
1	ZERE	VH3	CGG GAT TTC ATC TGA TGG AAC	VH3 GCC TCC TAC CAT TTT GCA ACA GCG TTC T JH3	TGT TGC AAA ATG GTA GGA GGC	61°C	169 bp
2	MIRO	VH3	ATT ACT AGT AGT ACT TTG	VH3 GTG GGA GCT TGG G JH4	CTC CCA AGC TCC CAC	50°C	153 bp

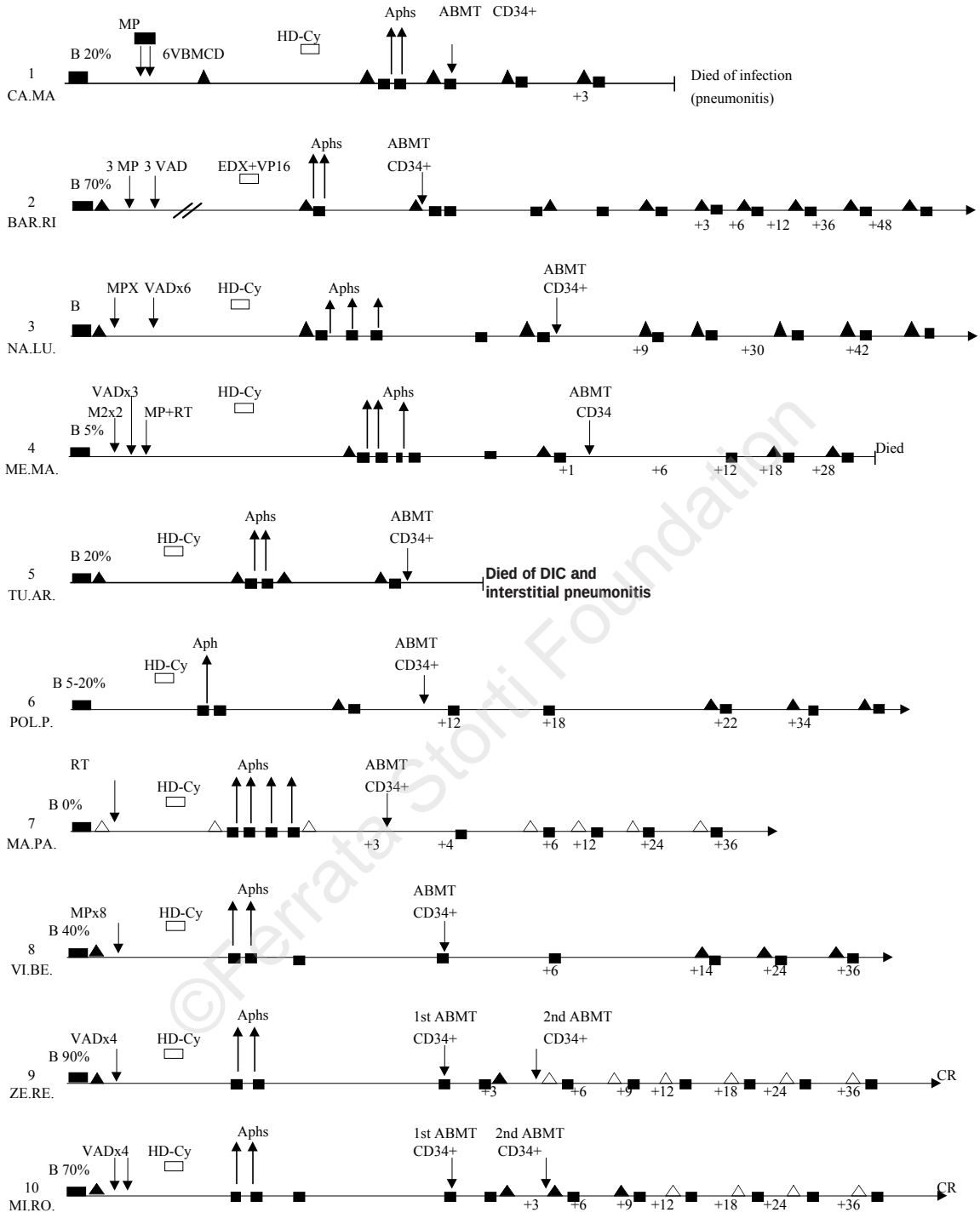

Figure 1. Schematic representation of clinical and molecular follow-up of the MM patients. Each patient is represented by a line: numbers under the lines show the months of follow-up.

ABMT = autologous bone marrow transplantation, CR = complete hematologic remission, HD-Cy = high dose cyclophosphamide. Open rectangle over the line represents HD-Cy. MP, MPX, VBMCD, VAD represents different chemotherapy schedules. The number before the chemotherapy represents the number of cycles of chemotherapy. RT= radiotherapy. Aphs= apheresis. Full and open triangles on the line represent IF positive and negative analyses, respectively. Full and open boxes on the line represent PCR CDR-III (ASO for #9 and 10) positive and negative analyses, respectively. The numbers followed by a plus mark (+) represent months of clinical follow-up after ABMT.

fluorescence and flow cytometry analysis. Interestingly, the percentage of plasma cells decreased after positive selection from 0.7±0.4% to 0.1±0.07% ($p<0.3$). This calculation indicated that tumor cells did not merely behave as *innocent bystanders* during the $CD34^+$ cell purification process, and that approximately 1-log depletion of myeloma cells was caused by the procedure. An additional 2-log purging resulted from the overall recovery of only 0.8% of MNCs.[9]

PBSC processing data, engraftment results, recovery and clinical outcome

The recovery of hematopoietic progenitor cells of most of our patients has already been reported along with engraftment and supportive-care data.[9] All MM patients achieved granulocyte engraftment in a median of 10 days and an unsupported platelet count $>20\times10^9$/L in a median of 15 days, respectively ($p>0.1$). The median time to reach $>50\times10^9$ Plt/L was 12 days for $CD34^+$ cells. One patient (#1) did not achieve platelet recovery (she had been reinfused with less then 2×10^6 $CD34^+$ cells/kg), and she died in the peritransplant period from interstitial pneumonia. Other clinical parameters, such as the duration of hospitalization, transfusion requirement, number of febrile days, documented infections and use of intravenous antibiotics were not different between this group and another group of MM patients transplanted with unmanipulated PBSC.[9] No patient received backup PBSCs or required platelet transfusion after discharge from hospital. Two patients (#9 and 10) were submitted to a second ABSCT and reinfused with $CD34^+$ cells in the same manner. Similar reconstitution results were obtained as in the first ABSCT. With a median time from reinfusion of 36 months (range 3-48), we have not thus far observed any late graft failure in patients who received purified $CD34^+$ cells. Seven patients are alive in hematologic remission: four of them had serum M protein assessable by immunofixation analysis, and two turned from serum IF positivity to negativity after 6 and 12 months from ABSCT, respectively. One was not secretory and three patients died, one from disease progression and body failure and two from interstitial pneumonia (Figure 1). All but one of the patients reinfused with $CD34^+$ cells are currently being treated with 3 U/m^2 of α-IFN (3 times a week) as maintenance therapy.

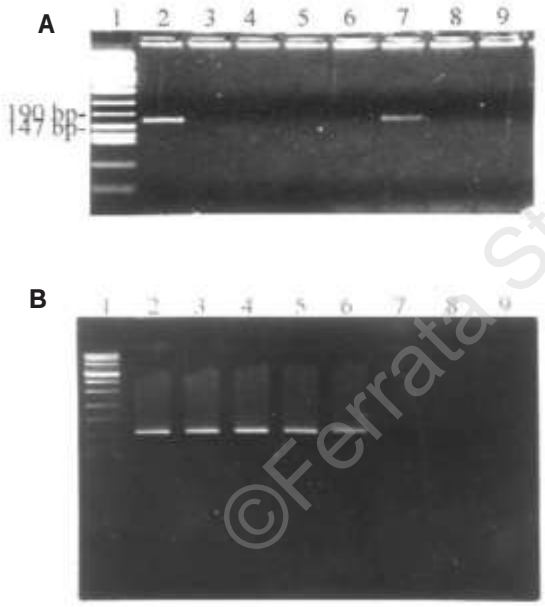

Figure 2. Molecular follow-up of patients #9 and #10 with patient-specific primers.
2a: Specificity of patient-specific PCR assay.
1 = molecular weight marker VIII (Boehringer Mannheim); 2 = patient #9 specific band, obtained with her patient-specific primers (PCR^+); 3 = patient #10 DNA amplified with patient #9 specific primer (PCR^-); 4 = negative control (DNA of a normal individual) (PCR^-); 5 = dH_2O (PCR^-); 6 = patient #9 DNA amplified with patient #10 specific primer (PCR^-); 7 = patient #10 specific band, obtained with his patient-specific primers (PCR^+); 8 = negative control (PCR–); 9 = dH_2O (PCR^-).
2b: Sensitivity of patient #9-specific PCR assay at DNA level.
1 = Molecular weight marker VIII (Boehringer Mannheim); 2 = 10^{-1} dilution assay; 3 = 10^{-2}; 4 = 10^{-3}; 5 = 10^{-4}; 6 = 10^{-5}; 7 = 10^{-6}; 8 = 10^{-7}; 9 = dH_2O

Evaluation of MRD on aphereses by DNA amplification of the CDR-III region of the IgH gene with consensus primers

Consistent with immunofluorescence studies, MRD was observed after stem cell purification in 8/10 patients' samples which were evaluated by PCR analysis of IgH gene rearrangement, with consensus primers as reported.[9] A schematic representation of clinical, laboratory and molecular outcomes is shown in Figure 1. The original clonal bands, identical in size to those observed in diagnostic BM samples were also found in each leukapheresis. In two patients (#9 and 10), the leukaphereses were only evaluated with patients' specific primers.

Molecular follow-up by PCR analysis of IgH gene rearrangement by patient specific primers

Patient-specific primers were generated to study the patient-specific IgH gene rearrangement in 2 patients (#9 and 10) who were IF negative 12 months after ABSCT (Figure 1). We were able to determine the CDR-III coding region by direct sequencing of PCR product generated by family specific primers. We generated the MM patients' specific primers (based on the CDR-II and CDR-III regions), and verified their patient-specificity (Figure 2a). We also assessed the sensitivity of our PCR assay at DNA level, which for each patient could detect at least one neoplastic cell diluted in 10^{-5}-10^{-6} negative cells (Fig-

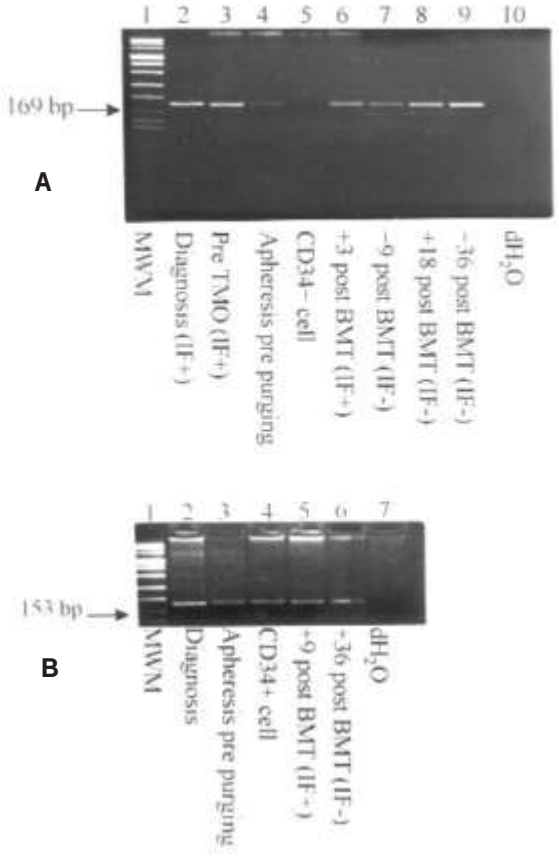

Figure 3. Molecular follow-up of patients #9 and 10 with patient-specific primers.
3a: specificity of patient-specific PCR assay.
1 = molecular weight marker VIII (Boehringer Mannheim); 2 = diagnosis (IF^+); 3 = pre-TMO (IF^+); 4 = apheresis pre-purging; 5 = $CD34^+$ cell; 6 = +3 post-BMT (IF^+); 7 = +9 post-BMT (IF^-); 8 = +18 post-BMT (IF^-); 9 = +36 post-BMT (IF^-); 10 = dH_2O.
3b: sensitivity of patient-specific PCR assay at DNA level.
1 = molecular weight marker VIII; 2 = diagnosis; 3 = apheresis pre-purging; 4 = $CD34^+$ cell; 5 = +9 post-BMT (IF^+); 6 = +36 post-BMT (IF-); 7 = dH_2O.

ure 2b). We found that although the stem cell purified fraction ($CD34^+$ cells) from patient #9 was negative for clonal rearrangement and that of patient #10 was positive, during follow-up both patients showed the presence of neoplastic cells in all the samples, even though they had become IF negative (Figure 3a and 3b).

Discussion

Circulating hematopoietic stem cells (PBSC) are generally believed to be involved by myeloma less frequently than BM harvests.[25] The use of PBSC in transplantation offers several advantages over BM stem cells, including a faster recovery of hematopoiesis. For these reasons, PBSC transplantation is being used in many centers for the treatment of MM patients after myeloablative therapy.[5,6] Indirect purging of neoplastic cells provided by positive selection of hematopoietic $CD34^+$ cells, has recently been proposed.[9,26,27] Our study confirms that the concomitant mobilization of plasma cells and hematopoietic progenitors is detectable at molecular level. Leukapheresis products were always contamined by myeloma cells. Other authors[12-25,28] showed similar contamination of PBSC collections by myeloma cells. The biological and prognostic significance of tumor cells present in PBSC collections is still unknown, as relapse may be caused either by re-growth of residual clonogenic cells *in vivo*, or by reinfused malignant cells.[29] Thus, removal of myeloma cells from PBSC autografts by positive selection of hematopoietic $CD34^+$ cells could have biological effects on PBSC outcome.[6,30]

Since the CD34 antigen is known not to be expressed on the surface of mature plasma cells in MM,[31,32] anti-CD34 antibodies may be clinically useful for isolating hematopoietic precursors for transplantation in MM patients with a reduced but not eliminated neoplastic clone.[26] It has been reported that in collections of 99.99% pure $CD34^+$ cells, obtained after using the combination of two methods of purification, IgH gene clonal rearrangement was not detectable by means of PCR based technology.[26, 32] We confirm this observation (in case #9 of our series), which reinforces the validity of using $CD34^+$ enriched cells as a source of possible uncontaminated cells for ABMT.[33] Reinfusion of autologous $CD34^+$ cells has been shown to reconstitute normal hematopoiesis in MM and other cancer patients treated with high-dose chemotherapy.[9]

The clinical study and follow-up of these MM patients also provides evidence that purified $CD34^+$ progenitors are capable of restoring bone marrow function in MM patients in the early phase of ABMT, as well as in the subsequent months. To date, no late granulocyte or platelet engraftment failure has been observed in any of our surviving patients, despite maintenance treatment with α-IFN (median follow-up: 36 months), and our study confirms that such a $CD34^+$ enriched cell fraction is capable of reconstituting stable hematopoiesis. Thus, the rapid and sustained engraftment, together with the documented depletion of tumor cells, shows that positive selection produces an MM-purging effect without apparent loss of engraftment potential.

As far as regards clinical outcome, two of the ten patients died of infection (pneumonia) in the first year after ABMT. We think that this complication is probably more related to the ABMT conditioning regimen (including TBI), than to the immunosuppression associated with the transplantation of the purified $CD34^+$ cells. In fact, we and others have recently reported that the immunologic reconstitution of patients transplanted with $CD34^+$ cells does not significantly differ from that of past patients who received unmanipulated stem cells.[34] Seven patients are in hematologic remission and two of them have

no IF evidence of disease.

Concerning molecular follow-up, we have recently developed an assay using patient-specific primers[12] in order to achieve better specificity and if possible higher sensitivity for our PCR based IgH clonal rearrangement assay. Our present patient-specific PCR analysis at RNA level detects one IgH clonal rearranged cell diluted in 10^5-10^6 non-neoplastic cells with absolute patient-specificity, even though some discrepancies in sensitivity could be present due to different annealing temperatures, length of primers, etc. We applied this PCR strategy in the two patients who achieved IF negativity, and we showed that both remained PCR positive for IgH clonal rearrangement during follow-up. As the contribution of contaminating MM cells in PBSC collection to clinical relapse is currently unknown, it is difficult to assess their role in MRD.[28,32]

We conclude that positively selected $CD34^+$ cells provide a cell suspension that can be safely used as supportive therapy for patients undergoing a myeloablative conditioning regimen. This procedure allowed restoration of persistently normal hematopoiesis in the majority of our MM patients.

Contribution and Acknowledgments

GM was the principal investigator: he designed the study and was responsible for ethical approval of the program; MC was responsible for funding and direct supervision. CT and MA set up the PCR procedures and drafted the paper. MB and MRM developed and carried out the cryopreservation procedures and flow cytometry assay. NT and AdV were responsible for cytogenetic studies, ST, MC and RML critically revised the manuscript and gave the final approval for its submission.

The order of authorship was made on the basis of the contributions given to the study.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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