Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry

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Background and Objectives. Minimal residual disease (MRD) studies are useful in multiple myeloma (MM). However, the definition of the best technique and clinical utility are still unresolved issues. The aim of this study was to analyze and compare the clinical utility of MRD studies in MM with two different techniques: allelic-specific oligonucleotide real-time quantitative PCR (ASO-RQ-PCR), and flow cytometry (FCM).

Design and Methods. Bone marrow samples from 32 MM patients who had achieved complete response after transplantation were evaluated by ASO-RQ-PCR, using TaqMan technology, and multiparametric FCM.

Results. ASO-RQ-PCR was only applicable in 75% of patients for a variety of technical reasons, while FCM was applicable in up to 90%. Therefore, simultaneous PCR/FCM analysis was possible in only 24 patients. The number of residual tumor cells identified by both techniques was very similar (mean=0.29%, range=0.001-1.61%, correlation coefficient=0.861). However, RQ-PCR was able to detect residual myelomatous cells in 17 patients while FCM only did so in 11; thus, 6 cases were FCM negative but PCR positive, all of them displaying a very low number of clonal cells (median=0.014%, range=0.001-0.11). Using an MRD threshold of 0.01% (10^{-4}) two risk groups with significantly different progression-free survival could be identified by either PCR (34 vs. 15m, *p*=0.04) or FCM (27 vs. 10m, *p*=0.05).

Interpretations and Conclusions. Although MRD evaluation by ASO-RQ-PCR is slightly more sensitive and specific than FCM, it is applicable in a lower proportion of MM patients and is more time-consuming, while both techniques provide similar prognostic information.

Key words: multiple myeloma, autologous stem cell transplantation, minimal residual disease, quantitative ASO-PCR, flow cytometry.

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Multiple myeloma (MM) is a malignant disease characterized by an increased number of clonal plasma cells in the bone marrow. High-dose chemotherapy followed by autologous peripheral blood stem cell transplantation is routinely used for the treatment of young MM patients and produces a high rate of complete remissions.[1-3] Although variable, the duration of such responses does not usually exceed three years and disease recurrence still remains the leading cause of death. This is due to the persistence of residual tumor cells, known as minimal residual disease (MRD), responsible for tumor relapse. In recent years, the study of MRD by flow cytometry or polymerase chain reaction (PCR) has proven to be of great value in predicting clinical outcome in different hematologic malignancies.[4-7] In MM however, both the clinical utility and the most suitable method for MRD detection remain controversial.

Investigation of MRD by qualitative PCR of VDJH rearrangements with allelic-specific oligonucleotides (ASO-PCR) has shown prognostic value in MM patients after autologous and allogeneic stem cell transplantation.[8-10] Nevertheless, some difficulties have hampered the use of this technique. Firstly, it is applicable in only two-thirds of cases because of the lack of clonal targets for amplification, caused by the presence of somatic mutations in IgH genes. In addition, the clinical utility of qualitative PCR in the setting of autologous stem cell transplantation has been modest,[8,9] because the sensitivity is so high that most cases remain PCR-positive regardless of their different outcomes.[11,12] Different strategies have been suggested to resolve these problems. The first problem could be overcome by using additional targets such as incomplete DJH rearrangements.[13] For the second problem, the use of real-time quantitative PCR with the same strategy (ASO-RQ-PCR) could be the best alternative, since this has provided accurate quantification of MRD levels.

However, although several groups have published technical results with real-time PCR,[14-17] the clinical application of this methodology is still relatively limited in MM since only three studies are currently available and all included less than twenty patients who had achieved complete remission.[18-20]

Very recently, multiparametric flow cytometry has been shown to be an attractive alternative approach to MRD monitoring in MM. This technique not only allows the number of plasma cells to be monitored but also distinguishes between myelomatous and normal plasma cells,[21] facilitating the monitoring of global changes in the plasma cell compartment of MM patients. As a disadvantage, the myeloma clone includes not only plasma cells but also less mature B cells, which are immunophenotypically different from plasma cells.[22] Nevertheless, dilution experiments have shown that immunophenotyping has a sensitivity of 10^{-4} to 10^{-5}, with several reports demonstrating its clinical utility in the setting of autologous transplantation.[23,24]

The aim of this study was to evaluate and compare the clinical value of measuring residual myeloma disease by ASO-RQ-PCR and flow cytometry in a series of uniformly treated MM patients who had achieved complete remission or near complete remission according to slightly modified EBMT criteria.

Design and Methods

Patients and response criteria

Patients included in this study had been treated with the GEMM2000 protocol designed by the Spanish GEM-PETHEMA group (*Grupo Español de Mieloma - Programa Español para el Tratamiento de Hemopatías Malignas*). This protocol consists of four cycles of VBCMP/VBAD followed by high dose melphalan and autologous peripheral blood stem cell transplantation. Patients not achieving complete remission (CR) with this scheme received a second transplant of either autologous or allogeneic stem cells following reduced intensity-conditioning. Response to treatment was assessed according to slightly modified EBMT criteria.[25] A complete response was defined by a negative immunofixation test in serum and urine (confirmed in two different samples obtained at an interval of 6 weeks), absence of soft-tissue plasmacytomas, a normal serum calcium concentration, stable skeletal disease, and less than 5% plasma cells (PC) in the bone marrow. A near complete remission was defined by the absence of myeloma protein on electrophoresis, but positive immunofixation and <5% plasma cells in bone marrow. Patients were eligible for inclusion in this MRD study if they had achieved complete remission or near-complete remission after the first transplant and bone marrow samples taken at diagnosis and 3 months after the transplant were available.

ASO-RQ-PCR

Sampling and DNA extraction

Genomic DNA from bone marrow samples at the time of diagnosis and 3 months after transplant was isolated using DNAzol reagent (MRC, Cincinnati, Ohio, USA). DNA was stored at –20°C.

PCR amplification and analysis of the IgH genes

Complete VDJH rearrangements were amplified using three multiplexed tubes containing VH-family-specific primers for FR1, FR2 and FR3 primers with a JH consensus primer. Amplification of incomplete DJH rearrangements was performed in two different tubes, one containing family-specific primers for DH1 to DH6 and the second tube with the DH7 primer, together with a consensus JH. Primers were newly designed and tested during the BIOMED-2 Concerted Action.[26] All reactions were carried out in 50 μL containing 0.1μg of DNA samples and 10pm of each primer. For heteroduplex analysis, PCR products were denatured at 94°C for 10 min and then cooled at 4°C for 60 min to induce duplex formation.[27] The hetero and/or homoduplexes generated were rapidly loaded on a 10% non-denaturing polyacrylamide gel in 1×TAE buffer, run at room temperature and visualized by ethidium bromide staining. In addition, the clonal nature of the PCR fragments was confirmed by Gene-Scanning analysis according to well-known procedures.[28]

Sequencing and CDR3 identification

Clonal products were eluted from the polyacrylamide gel and directly sequenced in an automated ABI 377 sequencer using Big-Dye terminators (Applied Biosystems, Foster City, CA, USA). In order to avoid nucleotide misinterpretations because of Taq errors, all products were sequenced twice using 5´VH, DH primers or 3'JH primers. Germline VH, DH and JH segments from complete VDJH rearrangements were identified by comparison with the V Base[29] *http://www.mrc-cpe.cam.ac.uk/DNAPLOT* and IGMT database[30,31] *http://imgt.cines.fr* using on-line *DNAPLOT* (MRC Center for Protein Engineering). DH and JH germline segments from incomplete DJH rearrangements were identified using the BLAST search in the DH-JH germline locus sequence (accession number EMB/X97051, *http://www. ncbi.nlm.nih.gov/blast/*).

Once the segments had been identified, the N-Region was highlighted for the ASO-primer design because of its high specificity for each rearrangement.

Design of the ASO-primer

All ASO-primers were designed with Primer Express version 1.0 (PE Biosystems) and OLIGO 6.1 software

(Molecular Biology Insights, Cascade, CO, USA) complementary to the VH-DH or DH-JH junctional regions and complied with the following recommendations: (i) primer-dimer formation must have a ΔG lower than -3.5 kcal/mol, (ii) the ASO primer should not have GC-rich 3' ends, (iii) melting temperature differences between the ASO-primer and its corresponding JH primer must be lower than 2°C, (iv) amplicon size must always be lower than 170 pb, and v) the melting temperature of the probe must be 5-10°C higher than that of the primers to ensure strong binding of the probe during the annealing phase.[32]

Evaluation of ASO-primer design

A qualitative PCR with the ASO-primer and the respective JH intronic primer was carried out with several dilutions of the patient's diagnostic sample as a positive control and buffy-coat DNA from healthy donors as a negative control. A single band of the expected size should be present in the patient's samples whereas nothing should appear in the buffy coat sample. In addition, dilutions of the original diagnostic sample had to provide the clonal band at the dilution of at least 10^{-3}-10^{-4} (*see below*).

Real-time quantitative PCR (RQ-PCR)

For the RQ-PCR, the method of Verhagen *et al*. was employed.[33] This method includes the use of an ASO forward primer, together with one out of six reverse primers complementary to the intron (JH1-JH6) and one out of three JH consensus probes (JH 1-2-4-5, JH3 and JH6) depending on the specific JH segment used in the rearrangement. Only cases meeting the criteria for matching primers and probes according to Gonzalez *et al*.[16] were included in the study. No specific probes were considered because this would imply an unacceptable increase in the costs. All reactions were carried out in a 25 µL final volume, containing 12.5 µL of 2×Taqman Universal Mastermix (PE Applied Biosystems, Foster City, CA,USA), including AmpErase uracil N-glycosylase (UNG), 300 nM of each primer, 200 nM of probe, and 600 ng of genomic DNA.

The two-step amplification protocol consisted of a 2 min incubation step at 50°C to digest PCR contamination products via UNG, 10 min at 95°C (inactivation of UNG, denaturation of target DNA, and activation of AmpliTaq Gold), followed by target amplification by 50 cycles of 10 s at 95°C and 30 s at 59-63°C (depending on each particular ASO-primer). Each ASO-primer was tested at different annealing temperatures, ranging from 59-63°C, in order to determine the maximum sensitivity and specificity for each particular assay. Fluorescence data were collected during the annealing-extension phase of every cycle, using the ABI PRISM 7700 Sequence Detection System containing a 96-well thermal cycler (PE Biosystems). All RQ-PCR experiments were performed in duplicate, except MRD samples that were processed in triplicate. The quality and quantity of the DNA were assessed by RQ-PCR of the albumin gene as previously described.[34]

Standard curve evaluation

To determine the amplification efficiency and the sensitivity of the method, diagnostic DNA was diluted in 10-fold steps into DNA from normal mononuclear cells down to 10^{-5}. The dilution series was subjected to RQ-PCR analysis together with appropriate positive and negative controls (water and mononuclear cells). The maximal sensitivity was defined as the last dilution of diagnostic DNA in which at least one of the duplicate dilution samples resulted in a positive fluorescent signal with a maximal cycle threshold (CT) value of 40 cycles.[33] To consider an experiment as acceptable, the Y-intercept had to be <40 and the slope higher than 3.0 and lower than 3.7. Based on the assumption that each cell contains 6pg of DNA, the addition of 600ng of DNA in each tube was considered to be equivalent to the addition of 100.000 cells. Calculations were corrected according to the percentage of tumor cells by flow cytometry. The maximal theoretical sensitivity was calculated to be between 10^{-5} and 5×10^{-5} (1 and 5 copies per tube, respectively).

Quantification of MRD by flow cytometry analysis

The number of plasma cells present in the bone marrow at diagnosis was assessed by flow cytometry using a validated immunophenotypic approach.[23] The same sample used for molecular studies was used for immunophenotypic analyses. The panel of monoclonal antibodies in quadruple combinations included: CD38/56/19/45, CD138/28/33/38 and CD20/117/138/38. Plasma cell phenotypic aberrations were identified at diagnosis and then used as patient-specific probes for follow-up analyses.

To increase the level of sensitivity of the technique, we used a two-step acquisition procedure in which up to 2×10^6 cells were acquired through a specific *live-gate* drawn on SSC/CD38^{+++}/CD138$^+$ cells. In all cases, an FL1/FL2/FL3 isotype-matched negative control CD38 for antigen-positive cells was used to evaluate specifically the autofluorescence level of the plasma cells.[23] For data analysis, Paint-A-Gate software (Becton Dickinson, San José, CA, USA) was used according to well established methods.[35] The main variable evaluated in this study was the percentage of phenotypically aberrant MM-plasma cells in the whole bone marrow.

Statistical methods

To estimate the statistical significance of the differences observed between means, the Mann-Whitney U and Kruskal-Wallis tests were employed, using the SPSS statistical software (SPSS Inc., Chicago, IL, USA).

Table 1. Clinical and biological characteristics of the patients.

	At diagnosis N=24	At transplant N=24
Age (years)	58±9.8	59±9.7
Hemoglobin (g/dL)	10.3±2.5	11.4±1.3
Platelets (x10⁹/L)	204±718	223±12
Leukocytes (x10⁹/L)	6.7±2	7±2.3
Creatinine >2 mg/dL	22%	4%
ECOG score ≥ 2	59%	8%
Extramedullary involvement	16%	0%
Presence of lytic lesions	79%	75%
Hemoglobin <8.5 g/dL	30%	17%
Lactate dehydrogenase >460 U/L	0%	0%
Albumin <3.5 g/dL	50%	29%
Monoclonal proteinuria	54%	0%
Calcium >11.0 mg/dL	18%	0%
$\beta_2$microglobulin > 6 mg/mL	29%	4%
C-reactive protein > 6mg/dL	19%	0%
13q monosomy (assessed by FISH)	47%	NA
S phase plasma cells >1.8%	29%	NA

Response Status	Pre-Transplant	Post-Transplant
Complete remission, negative IFX	17%	58%
Complete remission, positive IFX	13%	41%
Other	70%	0%

Results expressed as median±standard deviation. IFX, immunofixation.

The χ^2 test (cross-tabs, SPSS) was used for comparison of dichotomous variables between groups. The relationship between percentages of plasma cells detected by flow cytometry and RQ-PCR was evaluated through linear regression (Regression SPSS).

Survival curves were plotted according to the method of Kaplan and Meier, and compared using the log-rank test (survival SPSS). Those variables displaying a significant association with survival in these analyses (p<0.05) or those for which prior studies had demonstrated a prognostic value were included in a multiple regression analysis (Cox regression, SPSS). The model was tested both by including the variables in a continuous fashion (continuous model) and by grouping them into categories (dichotomous model). This analysis was carried out using the *ENTER method*, since the stepwise method could not be applied due to the scanty number of events occurring in this series.

Results

Patients' characteristics and applicability of techniques

Fifty-three patients were initially included in the study (all had achieved complete or near-complete remission following autologous peripheral blood stem cell transplantation and bone marrow samples at diag-

nosis and 3 months post-transplant were available for all of them). Five were directly excluded from the study because they did not display an immunophenotypic pattern different from that of normal plasma cells, and therefore could not be used for immunophenotypic MRD investigation (90% applicability). For the remaining 48 patients, 16 were lost due to pre-analytical problems: four cases due to degraded diagnostic DNA, three because of insufficient diagnostic DNA to construct the standard curve, and nine because of insufficient tumor cells at diagnosis to provide a good PCR product to be sequenced. Only 24 of the 32 cases remaining fitted the criteria to be valid in molecular studies for MRD investigations (applicability of 75%). The reasons for withdrawing these eight cases were that three did not amplify any IgH rearrangement, three lacked a long enough N-region to design an ASO-primer, and two had mutations either in the probe or in the JH intronic primer. Alternative primers using the somatically hypermutated region always provided non-specific amplifications in healthy samples. As shown in Table 1, the patients' characteristics were similar to those reported for other transplant series with good response. It should be noted that all but one patient had stage II/III MM.

ASO-RQ-PCR and flow cytometry

For ASO-RQ-PCR analysis, a VDJH rearrangement was used in 10 cases and a DJH rearrangement in the remaining 14. Clonotypic cells were undetectable by RQ-PCR in 7 patients – MRD-negative cases, while in the remaining 17 patients (71%) ≥0.001% tumor cells (≥1 myelomatous cell per 10⁵ residual normal hematopoietic cells) could be detected (MRD-positive). MRD status detected by RQ-PCR correlated with the immunofixation status. Thus, when immunofixation was used to define complete remission, 62% of patients had results consistent with those of RQ-PCR studies, but 34% of patients were found to have positive MRD by RQ-PCR but were immunofixation negative; on the other hand, there was one patient (4%) who was MRD-negative by RQ-PCR but did have a positive immunofixation test (Figure 1A). It is plausible that the immunofixation would have changed to a negative result later. Unfortunately, this patient died from delayed veno-occlusive disease four months after transplant. The median percentage of tumor cells present in the bone marrow three months after transplant in MM patients achieving complete remission was 0.043%, with a range between 0 and 1.61 (mean 0.29%, SD 0.51%).

In order to discriminate between patients with high or low MRD at this time point, we established a threshold level of 0.01% (presence of more or less than one tumor cell within 10⁴ normal cells): eleven patients had MRD ≤0.01% (low MRD) while thirteen had >0.01%
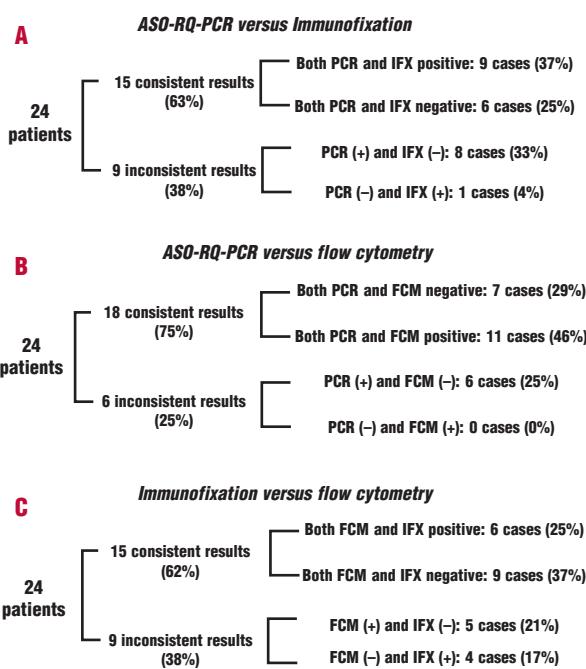
Figure 1. Comparison between minimal residual disease detection by RQ-PCR, flow cytometry (FCM) and immunofixation (IFX) three months after transplantation. In this figures, no cut-off point of 0.01% has been considered, so any detection of MRD is considered positive.

Figure 2. Progression-free survival curves according to the residual disease evaluation. A: based on MRD by RQ-ASO-PCR; B: based on immunofixation and C: based on MRD by flow cytometry. Discrimination between groups of risk according to MRD was made using a cut-off point of 0.01% (10^{-4}).

(high MRD). Regarding the predictive value of the MRD detected by RQ-PCR, the presence of a low MRD was associated with a longer progression-free survival. Thus, patients with less than ≤0.01% residual clonal cells displayed a progression-free survival of 34 months as compared to only 15 months for patients with a higher MRD level (p=0.042)(Figure 2A). Interestingly, other cut-off values, such as 0.1% were also predictive of the outcome of the patients (*data not shown*). It is important to note that there were two extramedullary relapses within the group with low MRD and that these were not predicted with this methodology. If these cases were excluded, the prognostic discrimination power of RQ-PCR was higher (p=0.019). By contrast, immunofixation status did not allow discrimination between two different risk categories (Figure 2B). As far as flow cytometry was concerned, immunophenotypically aberrant plasma cells were undetectable in 13 cases (54%, MRD-negative cases), while in 11 patients (46%) more than 0.01% tumor cells were detectable by flow cytometry (MRD positive cases). In comparison with ASO-RQ-PCR, the level of tumor cells detected by flow cytometry was very similar, ranging between 0 and 1.60 (mean 0.29%, SD 0.48%). Although six cases were negative by flow cytometry but positive by RQ-PCR (Figure1B), the number of clonal cells present in these cases was very low (median 0.014%, range 0.001-0.11%). Actually, as shown in
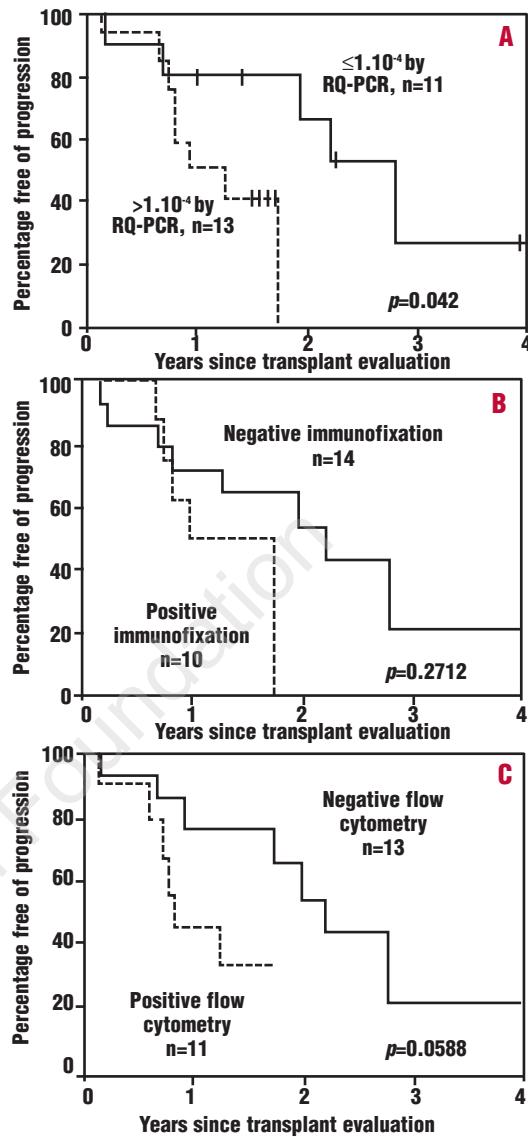
Figure 3, when the number of residual tumor cells detected by both techniques was compared in each individual case, the degree of correlation was very high (R=0,861). In addition, MRD detected by flow cytometry provided a very similar predictive value to that observed with ASO-RQ-PCR, with survival curves almost parallel (Figure 2). Thus, patients with <1 immunophenotypically aberrant plasma cells per 10,000 normal cells displayed a longer progression-free survival than those with a higher MRD (27 vs.10 months, p=0.05; Figure 2C). FCM did not provide a signficant value, although when considering all patients able to be followed (n=48) the p value was largely <0.05. Interestingly, as with RQ-PCR, other MRD threshold
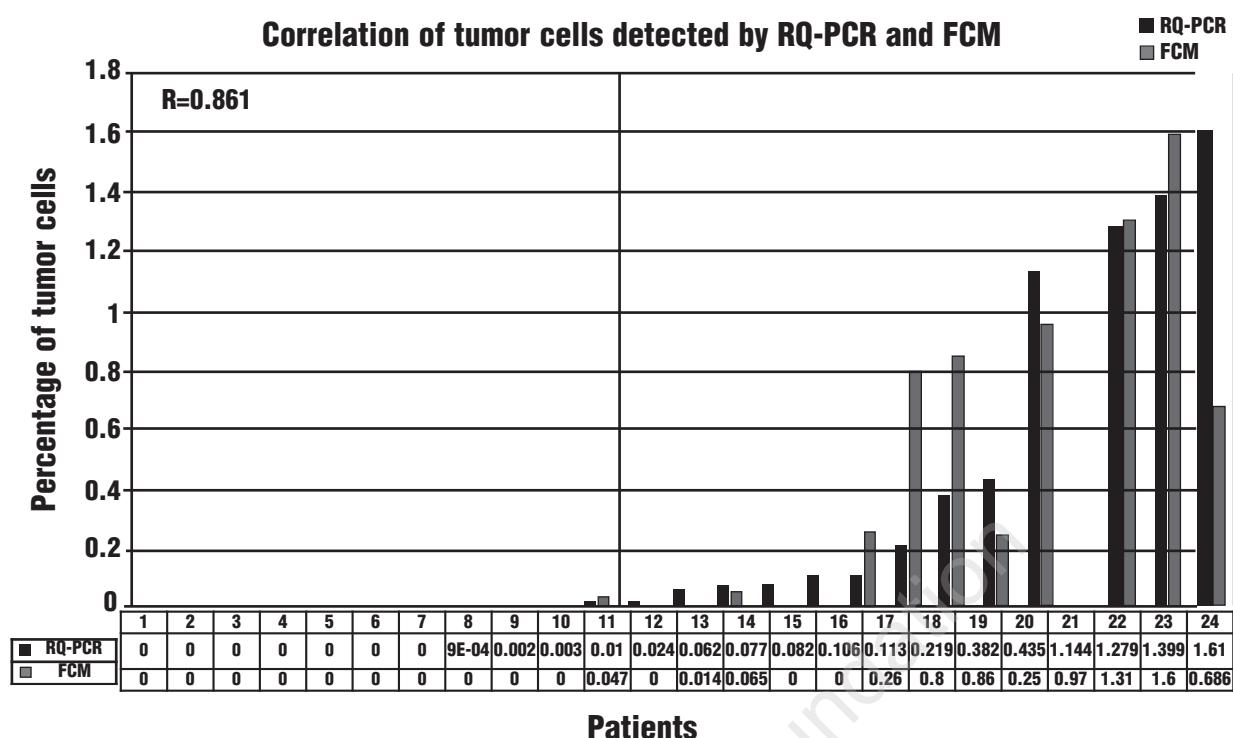
Figure 3. Relationship between flow cytometry (FCM) and ASO-RQ-PCR results of the number of tumor cells in the bone marrow of myeloma patients three months after transplantation.

values such as 0.1% were also predictive of progression-free survival. There were some patients with discrepant results between flow cytometry and RQ-PCR: three patients had $\leq 10^{-4}$ tumor cells detected by flow cytometry and $> 10^{-4}$ by RQ-PCR. Two of them had already relapsed while the other one is still free of progression. The two relapses were both conventional (M-component increase) and all patients were alive at the time of closing this study. Analyzing factors influencing the outcome of these patients, only two parameters were found to be associated with a shorter progression-free survival: advanced age (≥ 60 years) and high MRD level detected by RQ-PCR or flow cytometry. Other factors (including cytogenetics, β-2-microglobulin and percentage of S-phase plasma cells) did not achieve a significant predictive value, probably because of the strict patient selection criteria, especially that of including only patients achieving complete remission.

Discussion

We evaluated the utility of MRD investigations in bone marrow samples from MM patients achieving complete remission after high-dose therapy and autologous stem cell transplantation, in order to ascertain whether MRD assessment could contribute to a better definition of the quality of response and to determine its predictive value for relapse. For this purpose, we used and compared two different methodologies: ASO RQ-PCR and flow cytometry. This is the first study in which the clinical utility of ASO RQ-PCR is evaluated in a homogeneous series of transplanted MM patients, as well as the first time in which ASO-RQ-PCR is directly compared with flow cytometry. Our results show that both methodologies, ASO-RQ-PCR and FCM, may help to discriminate two risk categories of MM patients, based on the level of residual clonotypic or aberrant plasma cells ($>$ or 10^{-4}; high and low risk), showing significantly different outcomes. Using multiparametric flow cytometry, our group has previously demonstrated that, in MM patients achieving remission after autologous peripheral blood stem cell transplantation,[23,36] the persistence of >1 aberrant plasma cell within 10,000 total BM cells is associated with a shorter progression-free survival. In addition, the recovery of a favorable ratio between normal and myelomatous plasma cells predicts a better outcome after transplantation,[23] an observation recently confirmed by others.[37] The prognostic relevance of PCR investigations of MRD is less clear. Several groups, including our own, have found that the detection of IgH clonal rearrangements with low sensitive qualitative approaches such as heteroduplex and PAGE or fingerprinting, predicts a poorer outcome in patients undergoing autologous stem cell transplantation.[28,38-40] However, contradictory results have been reported when more sensitive tech-

niques, such as ASO-PCR, are used. Thus, while some groups have found that patients achieving PCR-negativity had prolonged progression-free survival after allogeneic or autologous transplantation,[9,10,41] other studies have found that clonotypic cells persist in virtually all MM patients after autologous stem cell transplantation, which prevents different risk populations from being differentiated.[11,12] This problem could be resolved by quantifying PCR results. However, only two quantitative studies with ASO-PCR have been reported in MM patients undergoing autologous peripheral blood stem cell transplantation. Bakkus et al.,[18] using a semi-quantitative ASO-PCR approach based on the limiting-dilution method, analyzed a total of 64 patients, although only 15 were in complete remission at the time of evaluation. A threshold MRD level of 0.015% was established as being optimal for distinguishing between risk groups of patients. Fenk et al.[19] used the RQ-ASO-PCR technique to evaluate the bone marrow obtained before transplantation in 11 MM patients undergoing autologous peripheral blood stem cell transplantation. An IgH/β_2 actin ratio >0.03% was able to predict a shorter progression-free survival independently of the clinical response. It should be noted that the cut-off values identified in these two studies are very similar to the 10^{-4} threshold used in the present study to separate high MRD from low MRD. However, in our study we found that several other less restrictive cut-off values (0.1%) also allowed risk categories to be established, indicating that the higher the MRD level the higher the risk of relapse. Interestingly, these cut-off points are the same as those identified by immunophenotyping.[23,36] Moreover, survival curves derived from both techniques (RQ-ASO-PCR and flow cytometry) were almost identical. Therefore, according to these results a possible goal for new treatment strategies for MM would be to reach a residual tumor load below 10^{-4}. Examining the advantages and disadvantages of ASO-RQ-PCR and flow cytometry techniques for MRD detection, the former has the advantage of identifying all clonotypic cells, including not only the plasma cells but also earlier precursor clonal B cells. However, a clonal result gives no definitive proof of malignant potential and it does not necessarily predict outcome.[42] Accordingly, six out of 13 RQ-PCR-positive patients did not relapse despite showing significant levels of tumor cells. By contrast, flow cytometry just focuses on the plasma cell compartment, while precursor tumor cells go undetected. This could explain why there were six patients in whom ASO-RQ-PCR detected very low numbers of tumor cells while flow cytometry was unable to detect any aberrant malignant cells. Despite this, as previously mentioned, both techniques yielded almost identical progression-free survival curves, using the same threshold level for discrimination of MRD risk groups. On the other hand, the applicability of flow cytometry (>90%) is significantly higher than that of PCR (≥75%) despite the use of highly standardized DNA V(D)J amplification methodologies[26] and the use of alternative targets such us DJH rearrangements.[16] Reasons for molecular failures were: short N-region (13%) and unamplifiable IgH rearrangement presumably due to somatic VH hypermutation (13%) or JH hypermutation (8%). Several experiments to improve this applicability failed to provide positive results. Thus, the main reason for molecular analysis failures was an inadequate diagnostic sample. Another important problem of molecular studies is that they are time and labor-consuming methodologies that provide a relatively modest advantage in clinical terms compared to flow cytometry. Thus, only two patients (10%) were allocated to the high-risk group by using ASO-RQ-PCR rather than flow cytometry.

In conclusion, investigation of MRD by quantitative ASO RQ-PCR in bone marrow of MM patients achieving complete remission after autologous stem cell transplantation provides relevant information on residual tumor load with a significant impact on the risk of relapse. However, other MRD techniques, such as multiparametric flow cytometry, yield similar prognostic information with the advantage of being easier and quicker and probably applicable to a higher number of patients. Thus, it is reasonable to think that flow cytometry will be the routine technique for assessing MRD in MM in clinical practice, but to reach this goal, additional studies including larger numbers of patients and longer follow-up are required. At the moment, we can say that real-time PCR and flow cytometry are complementary techniques in MRD evaluation for MM. Both techniques show that decreases in the bone marrow tumor load below 1 malignant cell per 10.000 total bone marrow cells could be used as a target for the definition of a molecular/immunophenotypic complete remission.

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