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ORIGINAL ARTICLE

Comparative analysis of minimal residual disease detection by multiparameter flow cytometry and enhanced ASO RQ-PCR in multiple myeloma

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Multiparameter flow cytometry (MFC) and allele-specific oligonucleotide real-time quantitative PCR (ASO RQ-PCR) are the two most sensitive methods to detect minimal residual disease (MRD) in multiple myeloma (MM). We compared these methods in 129 paired post-therapy samples from 22 unselected, consecutive MM patients in complete/near complete remission. Appropriate immunophenotypic and ASO RQ-PCR-MRD targets could be detected and MRD analyses constructed for all patients. The high PCR coverage could be achieved by gradual widening of the primer sets used for clonality detection. In addition, for 13 (55%) of the patients, reverse orientation of the ASO primer and individual design of the TaqMan probe improved the sensitivity and specificity of ASO RQ-PCR analysis. A significant nonlinear correlation prevailed between MFC-MRD and PCR-MRD when both were positive. Discordance between the methods was found in 32 (35%) paired samples, which were negative by MFC-MRD, but positive by ASO RQ-PCR. The findings suggest that with the described technique, ASO RQ-PCR can be constructed for all patients with MM. ASO RQ-PCR is slightly more sensitive in MRD detection than 6 – 10-color flow cytometry. Owing to technical demands ASO RQ-PCR could be reserved for patients in immunophenotypic remission, especially in efficacy comparisons between different drugs and treatment modalities.

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INTRODUCTION

The importance of attaining complete remission (CR) with first-line myeloma treatment has become apparent.[1,2] Accordingly, the development of new effective treatment strategies presents the challenge of finding the most precise means of comparing the efficacy of different therapies. In 2006, the International Myeloma Working Group published the consensus criteria for assessing response in multiple myeloma (MM), and categories of stringent CR, near CR (nCR) and very good partial remission were established.[3] International Myeloma Working Group updated the response and progression criteria in 2011, and the terms stringent CR , immunophenotypic remission (IR) and molecular remission (MolR) have been proposed for uniform reporting of clinical trials.[4]

So far, the various response assessment methods have been compared with only a few published trials.[5] In a study by Sarasquete *et al.*[6] of 32 MM patients in CR, multiparameter flow cytometry (MFC) proved applicable for minimal residual disease (MRD) measurement in 90% and ASO RQ-PCR in 75% of samples. ASO RQ-PCR showed better sensitivity in MRD detection. For the samples positive by both methods the correlation in MRD levels measured was good ($r = 0.861$).

Puig *et al.*[7] recently published an extensive study of 170 MM patients who had achieved at least partial response. Lack of clonality detection and unsuccessful sequencing or suboptimal ASO performance limited the applicability of PCR to only 42% of all cases. By adding data from a previous study they could, however, compare four-color MFC and ASO RQ-PCR in MRD detection in a cohort of 103 MM patients. All in all, persistent MRD could be identified in 46% of cases by MFC and in 54% of cases by ASO RQ-PCR. They found a significant correlation in MRD quantification by both techniques ($r = 0.881$, $P < 0.001$).

In summary of the findings of previous studies, ASO RQ-PCR is the most sensitive method available for MRD detection, the major drawback being, however, that it has proved applicable to only 42 – 75% of patients. MFC has been described as having higher applicability, but MRD analysis by these means has not reached the highest sensitivities attainable by ASO RQ-PCR. There is a need for a sensitive, well-standardized method with high applicability and feasible costs for routine laboratory use. The EuroFlow Consortium has published antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of plasma cell disorders in 2012 for more accurate identification of normal and aberrant plasma cells at diagnosis and follow-up samples.[8] Recently, a next-generation sequencing (NGS) method has been compared with ASO RQ-PCR and MFC in MRD detection in MM and B-cell disorders and has shown excellent concordance with

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PCR-MRD and MFC-MRD in 79.6 – 85% and 83%, respectively.[9–10] This new method does not require patient-specific reagents because it utilizes standard consensus primers and also has standardized sensitivity and quantifiable ranges. NGS methods may not only offer a more sensitive approach for tumor load measurement, but also information about mutations for personalized treatment. Although NGS was not able to identify an immunoglobulin (Ig)H clonotype in 2/10 MM patients owing to somatic hypermutation of IgH variable region,[9] a suitable clone for MRD follow-up was identified in 91% of MM patients.[10]

We applied an enhanced ASO RQ-PCR approach for MRD evaluation among consecutive patients reaching CR or nCR in a prospective study by the Finnish Leukemia Group in years 2009 – 2013, as previously reported.[11] In the present study, we compared MFC with ASO RQ-PCR in all the 129 paired follow-up samples currently available from these patients for MRD evaluation. In addition, the response assessment by MFC and ASO RQ-PCR is compared with immunofixation electrophoresis (IFE) and free light chain (FLC) ratio.

PATIENTS AND METHODS

Patients

Forty-seven patients with previously untreated, symptomatic MM, aged between 40 and 65 years were initially treated by induction therapy comprising four 3-week cycles of bortezomib 1.3 mg/m^2 intravenously on days 1, 4, 8 and 11 plus dexamethasone 40 mg on days 1–4 (all cycles) and on days 9–12 (cycles 1–2). Cyclophosphamide at a dose of 2 g/m^2 and granulocyte colony-stimulating factor were used for stem cell mobilization. Autologous stem cell transplantation (ASCT) was performed 24–48 h after conditioning with high-dose melphalan (200 mg/m^2). This study includes all 22 patients who achieved nCR or CR. Samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committees of the participating hospitals and by the Finnish Medicines Agency, and is registered with ClinicalTrials.gov, number NCT00861250. The design of the study has been published elsewhere.[11]

Clinical and laboratory evaluations were made at the time of registration, before each cycle, before mobilization and ASCT. Follow-up visits were scheduled at 3-month intervals during the first year, at 4 months during the second year and thereafter until progression. International uniform criteria were used to confirm progressive disease.[3,12]

Response assessment by protein analyses

Responses were assessed according to the international uniform criteria together with nCR response.[3,12] Response evaluation was based on serum and urine protein electrophoresis and immunofixation or on serum FLC levels when serum and urine paraprotein were not measurable. nCR was defined as an electrophoresis-negative but immunofixation-positive status together with bone marrow plasma cells $< 5\%$. Semi-automated agarose IFE was performed with Hydrasys LC or Hydrasys Isofocusing Systems and Hydragel 4 IF immunofixation gels (Sebia, Evry, France) as previously described.[13] Analyses were decentralized to five laboratories in the university hospitals of Finland. All laboratories participate regularly in IFE quality control survey.

FLC assay

The FLC assay was centralized in HUSLAB (Helsinki University Hospital Laboratories, Helsinki, Finland). The assays for kappa- and lambda-free light chains in serum were performed turbidimetrically in a Roche Modular P Analyzer (Mannheim, Germany) using reagents from The Binding Site Ltd (Birmingham, UK) (Freelite Human kappa Free Kit for Hitachi/Modular P and Freelite Human Lambda Free Kit for Hitachi/Modular P). Reference intervals were determined in HUSLAB by analyzing the kappa and lambda light chains of 124 Finnish adults (60 men and 64 women, aged 26–72 years) with normal results in serum capillary electrophoresis (albumin, alpha-1, alpha-2, beta-1, beta-2 and gamma fractions as well as total protein; all within their respective reference ranges, with no abnormal fractions). The reference intervals produced, representing 2.5 – 97.5% percentiles in the reference distribution, were as follows: serum kappa-free light chain 6.9–25.6 mg/l, serum lambda-free light chain 8.6–26.5 mg/l and free kappa/lambda ratio 0.52–1.40. In the clinical interpretation, ratios outside the reference interval were considered aberrant.

Response assessment by MFC

The MFC assay was standardized and decentralized to five laboratories in the university hospitals of Finland. Bone marrow samples were washed with PBS, stained with antibodies and analyzed with FacsCanto (Becton Dickinson, BD Biosciences, San Jose, CA, USA), FacsCanto II (Becton Dickinson, BD Biosciences) and Navios (Beckman Coulter Inc., Brea, CA, USA) flow cytometers for 6-, 8- and 10-color protocols, respectively. The percentages of 6-, 8- and 10-color protocols were 35%, 36% and 29% of samples, respectively. The expressions of CD138, CD38, CD45, CD19, CD56, CD27, CD28, CD117 and intracytoplasmic κ/λ were assessed in all samples, CD45 in 91% and CD20, CD81 and CD200 in 70% of samples. The sample was considered suitable for MRD analysis if plasma cells, normal or neoplastic, were detectable. Plasma cells were gated for analysis with CD138 and CD38 expressions and light-scatter properties. Myelomatous plasma cells and normal plasma cells were identified with different expressions of the above mentioned antigens according to European Myeloma Network guidelines.[14] Myeloma cells either expressed antigens not normally present on plasma cells or lacked antigens normally present on plasma cells. The most common immunophenotypic aberrancies observed in this study were (frequency in parenthesis): CD19 – (100%), CD56+ (77%), CD200+ (80%), CD81 – (62%), CD27 – (59%), CD117+ (41%), CD28+ (36%), CD20+ (27%) and 55% were clonal for κ, 45% for λ. MFC-MRD level was reported as a percentage of myeloma cells of total number of nucleated cells. The median number of events for MFC analyses was 790 000 (range, 95 831 – 2 963 089). At least 50 immunophenotypically abnormal plasma cell events were needed for MRD. MFC-MRD was assessed by patient-specific panels, and the sensitivity of the method was calculated by dividing 50 with the number of nucleated cells acquired, the median sensitivity being thus 0.0064% (range, 0.0017 – 0.052%). All five laboratories had concordant results in a national quality control survey.

Detection of clonal Ig gene rearrangements and ASO primer design

The molecular and ASO RQ-PCR assays were centralized in TYKSLAB (Turku University Hospital Laboratories, Turku, Finland). For molecular analysis unfractionated bone marrow samples were used for DNA extraction. The pretreatment bone marrow DNA samples were screened for clonal IgH, IgK and IgL rearrangements using BIOMED-2 multiplex primer sets and heteroduplex analysis on polyacrylamide gels.[15] Samples negative by the multiplex primers were further analyzed by singleplex PCR reactions with family-specific consensus primers.[16,17] Pooled buffy coat DNA from healthy blood donors was used as polyclonal control.

The clonal PCR products were cut from the polyacrylamide gel and sequenced in both directions by automated capillary electrophoresis sequencing (Applied Biosystems 3500Dx Series Genetic Analyzer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence analysis included identification of the V-, D- and J-genes, and (V) DJ junction analysis to design the ASO primer at the hypervariable complementarity determining region 3. For oligonucleotide melting temperature (*T*m) calculations an algorithm in the PrimerExpress (Applied Biosystems) was used. The initially designed ASO primer was first used in a preliminary real-time PCR together with the appropriate germline J-gene TaqMan probe and reverse primer.[18] In case where there was no amplification product or the amplification was not sufficiently specific compared to eventual amplification from polyclonal control, a patient-specific TaqMan probe for the V-gene and a reverse-oriented ASO primer were designed and used for MRD analysis.

Response assessment by ASO RQ-PCR

The pretreatment sample served as quantification standard for the follow-up samples. The sensitivity and quantitative range of the analyses were determined from according to EuroMRD guidelines.[19] However, putative low infiltration of clonal cells in pretreatment sample cause further challenge in MRD analysis of MM. Briefly, the pretreatment sample was diluted into polyclonal buffy coat DNA in a log-linear manner down to the level of 10^{-5}. The achieved sensitivity determined from the dilution series was further adjusted by taking into account the percentage of myeloma cells in the pretreatment sample assessed by flow cytometry, and the detection limit was specified individually for each patient and sample (Table 1).

Table 1. Detection of clonal Ig rearrangements in the pretreatment sample

Patient no.	*Clonal cells in the aspirate (%)*[a]	*IgH multiplex*	*IgK multiplex*	*IgL multiplex*	*IgH singleplex*	*Selected ASO target*	*Individual TaqMan probe*	*Sensitivity of ASO RQ-PCR analysis (%)*
1	2.1	–	–	–	+	VDJ (IgH)	+	0.0004
2	10	+	+	–	n.d.	VDJ (IgH)	+	0.001
3	3.5	–	+	+	–	VJ (IgK)	–	0.007
4	1.7	+	n.d.	n.d.	n.d.	VDJ (IgH)	+	0.001
5	1.39	+	n.d.	n.d.	–	VDJ (IgH)	+	0.0007
6	21.5	–	+	+	+	VDJ (IgH)	+	0.001
7	6	+	+	+	–	VDJ (IgH)	+	0.001
8	8	+	–	–	+	DJ (IgH)	–	0.0004
9	20	+	n.d.	n.d.	+	DJ (IgH)	–	0.001
10	20	–	+	+	+	VJ (IgL)	+	0.001
11	8.4	+	n.d.	n.d.	–	VDJ (IgH)	+	0.001
12	4.3	+	+	n.d.	+	DJ (IgH)	–	0.0004
13	9.1	+	n.d.	n.d.	n.d.	DJ (IgH)	–	0.001
14	26	+	n.d.	n.d.	n.d.	DJ (IgH)	–	0.003
15	0.6	+	–	–	n.d.	VDJ (IgH)	+	0.0006
16	5	+	+	+	n.d.	VDJ (IgH)	-	0.003
17	5	+	+	+	n.d.	VDJ (IgH)	+	0.001
18	6	+	+	+	+	DJ (IgH)	–	0.0006
19	0.16	–	–	–	+	DJ (IgH)	–	0.0004
20	7.5	+	–	–	n.d.	VDJ (IgH)	+	0.0004
21	11	+	n.d.	n.d.	+	VDJ (IgH)	+	0.001
22	0.6	+	n.d.	n.d.	–	VDJ (IgH)	+	0.0006
Summary	0.16 – 26%	17/22 (77%)	9/15 (60%)	7/13 (54%)	9/14 (64%)		13/22 (55%)	0.0004 –0.007

Abbreviations: ASO, allele-specific oligonucleotide; Ig, immunoglobulin; n.d., not done; RQ-PCR, real-time quantitative PCR. The proportion of clonal plasma cells in the bone marrow aspirate sample was determined by flow cytometry. IgK multiplex, IgL multiplex and IgH singleplex PCR reactions were done where the initial IgH multiplex reactions were negative, or whether the initially designed ASO primer performed poorly. For about half of the patients the best performing analysis was obtained by a reverse-oriented ASO primer and individually designed TaqMan probe. [a]Of all nucleated cells as determined by flow cytometry.

The albumin gene was used for normalization of the ASO RQ-PCR results. 0.5 μg of follow-up sample DNA was used in each of the three replicate analyses. The maximum sensitivity achievable was thus ~0.0004% (4E – 6) based on the total amount of 1.5 μg of DNA, corresponding to ~240 000 nucleated cells. Molecular MRD analyses were carried out according to the EuroMRD guidelines both in the PCR set up and in interpretation of data.[19] Molecular progression was defined as an at least 10-fold (1 log) increase in MRD in ASO RQ-PCR between consecutive bone marrow analyses.

Statistics

Statistical analyses for the relationship between MFC-MRD and PCR-MRD were performed with R statistical software (R Core Team 2013, R Foundation for Statistical Computing, Vienna, Austria), version 2.15.2. The exponential growth function was used to calculate the value of nonlinear correlation between MFC-MRD and PCR-MRD results. The possible predictive impact of IFE and FLC ratio for IR and MolR was analyzed using the logistic regression model with random effects. Progression-free survival (PFS) was analyzed by the Kaplan–Meier method, and the differences for statistical significance between curves were tested with the two-sided log-rank test. PFS was measured from the ASCT date to the date of progression or death. For PFS analyses SPSS software (version 19.0; SPSS, Chicago, IL, USA) was used.

RESULTS

PCR amplification and sequencing of Ig genes

Results of the amplification of Ig genes are summarized in Table 1. Ig clonality could be detected in the pretreatment sample in all of the 22 patients who achieved nCR or CR. Where the initially recognized IgH clonality could be sequenced and a fully functioning ASO primer designed, no further clonality screening was undertaken. If necessary, further analyses included IgK and IgL multiplex PCRs and IgH singleplex PCRs.

The proportion of clonal plasma cells in the bone marrow aspirate collected and stored before treatment varied between 0.16 and 26% as evaluated by flow cytometry. In the sample with the lowest proportion of clonal cells (0.16%) all multiplex PCRs with Biomed primer sets yielded negative results. A specific clonal product could only be identified by the class-specific IgH singleplex PCR. This also applied to another sample with a low (2.1%) proportion of clonal plasma cells. Singleplex PCRs were performed for an additional six patients in order to produce better quality samples for sequencing, but for all of these the clonal product was also identifiable in multiplex PCR analyses.

At least one clonal Ig gene rearrangement could be successfully sequenced for each patient. ASO primers were designed for both rearrangements in cases where both heavy chain and light chain rearrangements were sequenced. The primer giving the best sensitivity was then chosen for follow-up analyses. For 20 patients IgH was chosen as the ASO target, for 1 patient each IgK and IgL. More detailed data on the gene segment repertoire by rearrangement are given in Table 2.

ASO primer design and testing

For nine patients the standardized method (originally designed for acute lymphoblastic leukemia) with one specific forward primer and a J-gene-specific consensus TaqMan probe yielded an assay sufficiently sensitive and specific for MRD assessment. For 13 patients the standard assay was either not sensitive enough or did not function at all. For all of these patients an alternative approach with a reverse-oriented allele-specific primer on the junctional sequence, individually designed TaqMan probe and forward consensus primer on the V-gene, yielded an assay sufficiently sensitive and specific for MRD assessment. One patient (patient no. 22) did not wish to have any follow-up bone marrow specimens drawn, and no molecular follow-up was undertaken

Table 2. Gene segment repertoire by rearrangement

IGHV-J						*IGHD-J*				*IGKDEL*	
IGHV		*IGHD*		*IGHJ*		*IGHD*		*IGHJ*			
1	2	1	1	1	—	1	2	1	—	Biallelic	1
2	1	2	3	2	1	2	3	2	—	Intron-RSS	3
3	9	3	8	3	3	3	—	3	—	IGKV1	1
4	5	4	1	4	8	4	—	4	4	IGKV2	1
5	—	5	–	5	3	5	3	5	4	—	—
6	—	6	4	6	2	6	2	6	2	—	—
7	—	7	—	—	—	7	—		—	—	—

In addition to rearrangements described in the table IgK KV-JK rearrangement was detected in four patients (two IGKV3-JK5, one IGKV3-JK1 and one IGKV2-JK2 rearrangement). IgL rearrangement was detected in two patients (one IGLV3-JL2/3 and one IGLV2-JL2/3).

for this patient. The median sensitivity was 0.001%, range 0.0004 – 0.007% as determined from dilution series of pretreatment sample into negative control DNA. The EuroMRD criteria for sensitivity estimation were strictly followed (Table 1).

Comparison of ASO RQ-PCR, MFC, IFE and FLC in response evaluation

Thirteen (59%) of the 22 patients with nCR/CR achieved MolR on intention-to-treat analysis and 4 more patients (77%) showed MFC-MRD negativity, but were PCR-positive. In these patients a total of 129 consecutive samples from the time point of transplantation were collected and analyzed with a median follow-up of 16 (range, 6 – 28) months. MFC and PCR were concordant in 75% of analyses, both positive in 29% or both negative in 46% of analyses. In 35% (32/91) of MFC-negative samples MFC was negative with a median sensitivity of $< 0.006\%$ (range, 0.05–0.001%), but PCR detected MRD with a median level of 0.003% (range, 0.02–0.0004%). Only one sample was MFC positive, 0.005%, but PCR negative (sensitivity $< 0.001\%$). A comparison with positive and negative PCR and MFC results of all 129 samples is given in Table 3. The relationship of all MFC-MRD and PCR-MRD results are illustrated in Figure 1, where a coefficient of determination $r^2 = 0.72$ ($P < 0.001$) is seen.

Neither negative serum nor urine IFE nor normal FLC ratio was statistically significant predictors for MolR ($P = 0.44$ and $P = 0.06$, respectively). Their predictive value for IR was even lower. The distribution of MFC- and PCR-MRD results between IFE-negative and -positive samples is illustrated in Figure 2, where also the numbers of abnormal FLC ratios in each group are given. Serological concordance between IFE and FLC ratio was 63%.

Prognostic value of MRD monitoring by ASO RQ-PCR and MFC

We also updated survival by MRD load. Both MRD methods showed statistically significantly prolonged PFS for MRD-negative patients, with an estimated median PFS of 48 months in both groups, compared with a PFS of 16 months in PCR-positive ($P = 0.004$) and to 13 months in MFC-positive ($P = 0.011$) patients (Figure 3). The prognostic value of our ASO RQ-PCR method was also analyzed in the group of 16/22 (73%) patients in CR, and showed statistically significant PFS for MRD-negative patients (Figure 4).

Table 3. The comparison of all PCR-pos/neg and MFC-pos/neg samples from patients who reached nCR/CR

PCR/MFC	*MFC neg* N = *91(%)*	*MFC pos* N = *38(%)*	*Total* N = *129(%)*
PCR neg	59 (65)	1 (3)	60 (47)
PCR pos	32 (35)	37 (97)	69 (53)

Abbreviations: CR, complete remission; MFC, multiparameter flow cytometry; neg, negative; nCR, near CR; pos, positive.

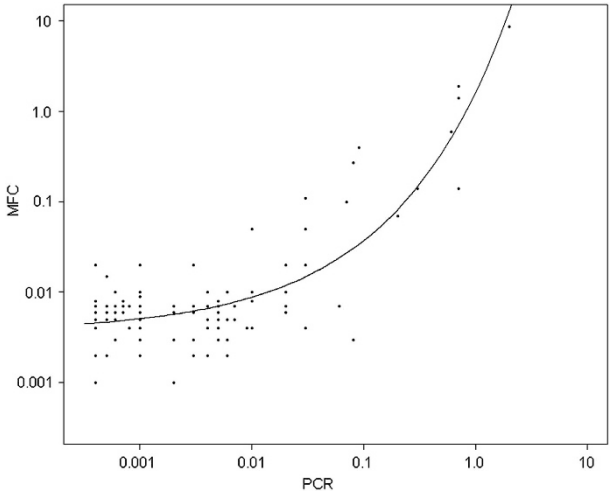

Figure 1. The relationship between PCR-MRD (%) and MFC-MRD (%) results ($n = 122$), positive or negative, of 22 patients in nCR/CR from ASCT to the median follow-up of 16 (range, 6–28) months, $r^2 = 0.72$ ($P < 0.001$). The relationship between PCR-MRD (%)- and MFC-MRD (%)-positive results was $r^2 = 0.74$ ($P < 0.001$).

DISCUSSION

Several ongoing studies on different myeloma treatments indicate promising impact on future treatment strategies. Assessment of responses calls for a practical and reliable method to detect MRD, reflecting the depth of response. Both MFC and PCR have been investigated in this context. According to earlier observations, there is a correlation between the two methods, but PCR seems to be more sensitive by roughly one logarithm.[6] Nonetheless, a clone-specific primer is mandatory for PCR assays, and the success rate in designing such clone-specific and sensitive assays has varied from 42 to 100%.[6–7,11,13,20]

Puig *et al.*[7] discuss the reasons for the low feasibility rate of ASO RQ-PCR in MM. They point out that feasibility studies should include unselected MM patients, and the universally accepted EuroMRD guidelines for interpretation of ASO RQ-PCR should be followed. Somatic hypermutations are characteristic of MM and they may hamper clonality detection, sequencing success and

ASO performance. The authors used the standardized method of ASO RQ-PCR designed for acute lymphoblastic leukemia using only one specific forward primer. However, they suggest that alternate approaches for assay design might improve the feasibility rate.

As highlighted in a study by Martinez-Lopez *et al.*[21] clonal rearrangement can actually be detected at the molecular level in a very high percentage (91.5%) of all MM patients. This percentage should be even higher if Ig light chain rearrangements are considered, in addition to IgH and Kde rearrangements. By sequencing all these rearrangements and using alternate locations for consensus primers and, if necessary, even individualized TaqMan probes to cover cases with somatic mutations in the V-gene, it should be possible to attain a much higher feasibility rate. We have taken three steps to enhance the feasibility of ASO RQ-PCR[11] in our patient cohort of consecutive and unselected symptomatic MM patients in nCR/CR, namely (1) use of singleplex Ig consensus primers in addition to the multiplex primer approach, (2) inclusion of the light chain rearrangements for clonality detection and (3) if necessary, use of a reverse-oriented allele-specific primer and individually designed TaqMan probe in the ASO RQ-PCR analysis.

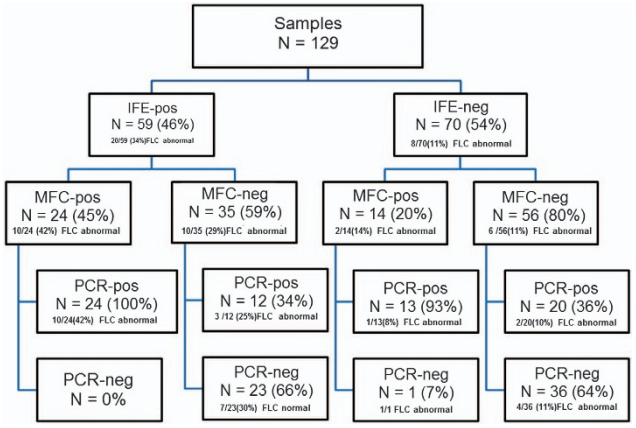

Figure 2. All of the 129 follow-up samples from patients who reached nCR or CR were investigated by the protein assays IFE and FLC, by flow cytometry and by ASO RQ-PCR. The distribution of results over different categories is shown in this table. Among these patients IFE and FLC did not correlate with the response status measured by MFC or ASO RQ-PCR.

With applicability rates of ASO RQ-PCR and MFC proving roughly equal, the remaining question concerns the sensitivity of these two methods. Improvement in the sensitivity of MFC should be possible by increasing the number of events under analysis. Even if the quality or available amount of bone marrow sample imposes limitations it should be possible to improve the sensitivity by increasing the number of simultaneous fluorescent colors in the analysis. Protocols and equipment are available for 6, 8 or even 10 colors, whereas most studies published so far have been made on 4-color protocols. Further, the sensitivity of ASO RQ-PCR can be improved by increasing the amount of DNA analyzed (for example, by adding replicate PCR reactions). Molecular analysis by PCR requires that DNA from the pretreatment sample has been stored for eventual later construction of the ASO RQ-PCR analysis. One distinct advantage of PCR-based analysis in comparison with flow cytometry is that long-term storage and later centralized analysis of samples can be arranged in multicenter studies. The same applies to the NGS method, in which the best achievable sensitivity (10^{-6}) can also be limited by an insufficient amount of DNA.

The methodological efforts of three-step ASO RQ-PCR increase the costs of analysis by ~30%. This method would be suitable especially for multicenter myeloma studies to study deep remission status among patients in CR. It is possible that the sensitivity of MFC of $< 10^{-4}$ could also be improved to $< 10^{-5}$–10^{-6}

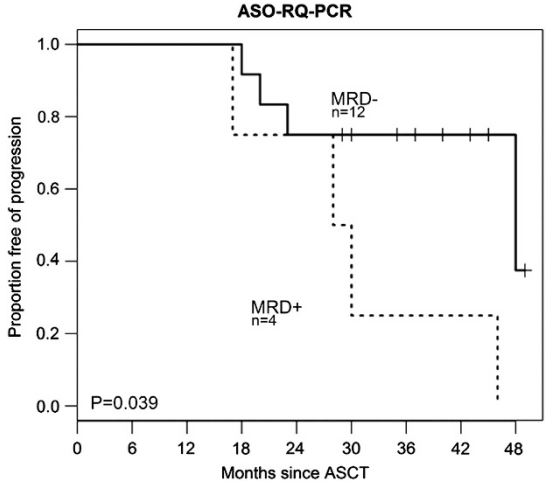

Figure 4. PFS of CR patients according to molecular response.

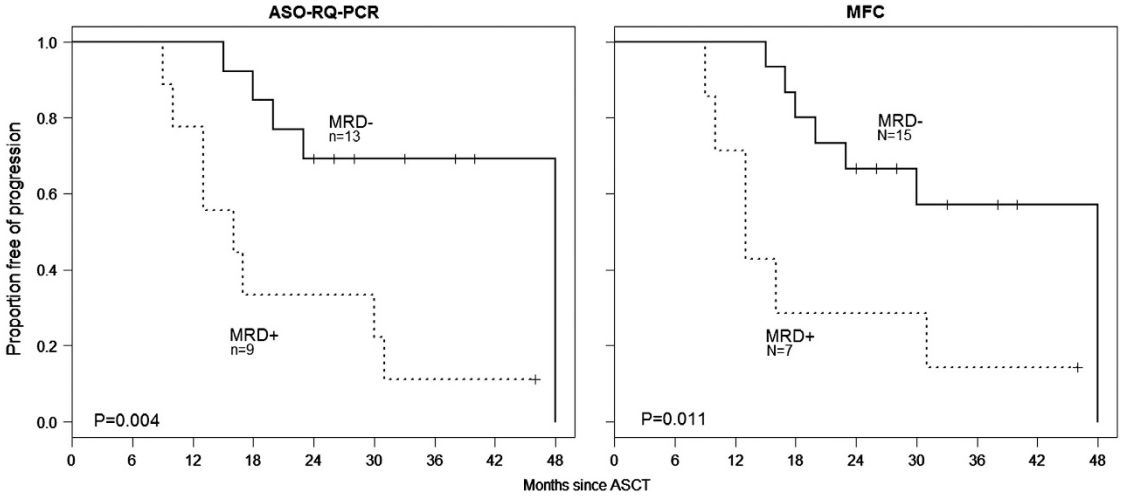

Figure 3. PFS according to MRD by ASO RQ-PCR and MFC of patients who achieved nCR/CR.

by using $8-10$-color MFC and analysis of $0.5-1\times10^7$ cells,[22] but theoretically the sensitivity of MFC will not reach the sensitivity and specificity of ASO RQ-PCR on any similar amount of sample materials analyzed. The fundamental difference between these two methods is that MFC usually requires $10-50$ residual clonal cells for MRD detection, whereas an optimal ASO RQ-PCR and NGS method may detect even a single residual clonal cell. It may thus be predicted that on any amount of sample material analyzed ASO RQ-PCR or NGS will have about 10-fold higher sensitivity than MFC. For rearrangement detection, NGS may be the method of choice as it offers the advantage of clone quantification in the pretreatment sample and wide screening of possible rearrangements.

The weakness of our MFC method could be decentralizing, which, however, was mandatory for use of fresh samples. This study was performed before the publication of EuroFlow standardization for MFC in plasma cell diseases,[8] but MFC was performed by the guidelines of European Myeloma Network[14] in all five centers. The authenticity of MFC-negative results could be demonstrated with the PFS difference between MFC-positive and -negative patients (Figure 3). Thus, novel standardized MFC should be compared with our enhanced ASO RQ-PCR regarding applicability, costs and sensitivity, and reflected on the outcome of patients.

The FLC ratio was not useful for response estimation in the present study. Patients in nCR/CR IFE and FLC assays are utilized close to or below their reproducible sensitivity for paraprotein detection. Random factors may thus contribute to the result being interpreted as either negative or positive, and it may be no surprise that results are discordant to each other and to PCR-MRD or MFC-MRD. This observation is in agreement with earlier studies.[23–29]

In conclusion, with our enhanced ASO RQ-PCR we were able to offer PCR follow-up for all patients by the protocol with a good sensitivity of MRD. ASO RQ-PCR was slightly more sensitive than our standardized, $6-10$-color MFC. However, immunophenotypic response showed a significant nonlinear correlation with molecular response, $r^2=0.72$ ($P<0.001$). MRD evaluation by ASO RQ-PCR thus served as a better biological indicator of treatment efficacy among these very favorably responding patients, but this needs to be confirmed in larger studies comparing this enhanced ASO RQ-PCR with new MFC methods. This enhanced three-step ASO RQ-PCR will probably not be implemented in daily practice owing to the laborious process, but an MRD method with superior sensitivity and applicability is still needed in clinical trials when declaring any novel treatment more efficient than the other.

CONFLICT OF INTEREST

RS has received honoraria from Genzyme, Sanofi, Celgene and Janssen-Cilag, and research funding from Janssen-Cilag and Celgene; KR has received honoraria from Celgene and Janssen-Cilag; MP has received honoraria from Celgene; PA has received honoraria from Celgene and Janssen-Cilag; VK has received lecturing honoraria from Novartis and an educational grant from Novartis and Celgene; TTP has received educational grants from Celgene, Bristol-Myers-Squibb and Novartis; TL has received an educational grant from Bristol-Myers-Squibb; VJ has received an educational grant from Novartis and the remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

RS, KR, VK, TTP designed the study, VK, TL, VJ performed the ASO RQ-PCR analytics, TTP, TLP, VH, PM, SS performed the MFC analyses, LU standardized the FLC assay, PA, MP, KR, RS provided study material or patients, TS and RS performed statistical analyses, RS wrote the manuscript, VK and KR revised the manuscript and all authors edited and approved the manuscript.

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