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Levels of minimal residual disease detected by quantitative molecular monitoring herald relapse in patients with multiple myeloma

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ROLAND FENK
MUHARREM AK
GUIDO KOBBE
ULRICH STEIDL
CAROLIN ARNOLD
MARK KORTHALS
ALI HÜNERLITÜRKOGLU
ULRICH-PETER ROHR
SLAWOMIR KLISZEWSKI
ALF BERNHARDT
RAINER HAAS
RALF KRONENWETT

A B S T R A C T

Background and Objectives. Detection of minimal residual disease (MRD) has helped to improve the treatment of patients with leukemia. At present MRD testing in patients with multiple myeloma (MM) is not applied as a standard diagnostic or prognostic method.

Design and Methods. Immunoglobulin heavy chain (IgH) polymerase chain reaction (PCR) using patient-specific TaqMan probes together with LightCycler technology was performed to quantify minimal residual disease in MM. Relative levels of clonotypic cells were assessed as IgH/2β-actin ratios with a sensitivity of 10^{-4} to 10^{-5}.

Results. Following stem cell transplantation, a significant reduction of clonotypic cells was observed in bone marrow (BM) and peripheral blood (PB) samples of 11 patients, comparing pre-treatment values with those of best response (median: 13% to 0.09% and 0.03% to 0%, respectively). In 5 patients with ongoing clinical remission IgH/2β-actin ratios remained stable at a low level, while in 6 patients an increase to 2% in BM and 0.4% in PB was associated with progression of the disease. In 4 of these 6 patients the increase of clonotypic cells in PB was detectable a median of 3 months (range: 0.5-6) before relapse. Furthermore, time-to-progression of patients with pre-transplantation IgH/2β-actin ratios > 0.03% in BM was significantly shorter than that of patients with lower MRD levels.

Interpretations and Conclusions. MRD in patients with MM can be quantified reliably using TaqMan chemistry adapted to the LightCycler system. Residual tumor cell levels before transplantation as well as results of sequential molecular monitoring are predictive of relapse.

Key words: multiple myeloma, immunoglobulin H, molecular monitoring, minimal residual disease, real-time quantitative PCR.

From the Dept. of Hematology, Oncology and Clinical Immunology University of Duesseldorf, Germany.

Correspondence: Dr. Roland Fenk, Dept. of Hematology, Oncology and Clinical Immunology, University of Duesseldorf, Moorenstr. 5, 40225 Duesseldorf, Germany. E-mail: fenk@med.uni-duesseldorf.de

Polymerase chain reaction (PCR) using allele-specific oligonucleotide (ASO) primers complementary to the unique sequence of the immunoglobulin heavy chain gene (IgH) is a widely used technique for detecting residual tumor cells in patients with B-cell malignancies.[1,2] The existence of residual tumor cells in samples of peripheral blood (PB) and bone marrow (BM) measured by qualitative IgH PCR has a prognostic value in patients with MM,[3-8] acute lymphoblastic leukemia (ALL)[9-11] and low-grade non-Hodgkin´s lymphoma such as follicular lymphoma, mantle cell lymphoma and chronic lymphatic leukemia (CLL).[12-15] Meanwhile, quantitative PCR methods are available and have shown that the extent and changes of residual tumor cell infiltration are of clinical relevance. In childhood and adult ALL, quantitative MRD after transplantation can define groups of patients at different risks of relapse[16,17] and in patients with CLL real-time PCR is used to assess response to novel therapies.[18,19]

In patients with MM quantitative MRD monitoring using IgH PCR has been used in studies to quantify purging efficiencies[20-23] and to examine the prognostic value of MRD after transplantation.[24-27] At present, the prognostic value of the amount of residual tumor cells in PB, BM or stem cell harvests and its changes over time is still not clear. Limiting dilution PCR[28] and real-time quantitative (RQ)-PCR[29-32] for IgH have been established to quantify clonotypic cells. Two different detection systems are used for RQ-PCR: the ABI Prism Sequence Detection System (SDS) which uses one hydrolysation probe labeled with a reporter and a quencher dye (Taqman probe)[33] and the LightCycler system which is based on the use of two hybridization probes, each

Table 1. Patients characteristics.

No.	age	sex	type	stage	ß2	therapy	response	EFS
1	51	f	IgGκ	II A	1.2	auto + allo	CR	414†
2	52	m	IgAκ	III A	4.8	auto + INF	CR	467+
3	65	m	IgMκ	III A	1.9	auto + INF	PR	690+
4	53	m	IgGκ	III A	2.1	auto + Thal	PR	607+
5	45	m	IgGκ	III A	2.3	auto + INF	MR	423+
6	45	m	IgGκ	III A	1.5	auto	CR	309 PD
7	55	m	IgG/Aλ	III A	4.3	auto + Thal	CR	274 PD
8	41	m	IgGκ	III A	3.0	auto + INF	CR	112 PD
9	45	m	IgGκ	III A	1.5	allo + Thal	PR	508 PD
10	62	m	IgG	II A	2.4	auto + Thal	PR	413 PD
11	48	m	IgGλ	III B	3.4	auto + INF	PR	334 PD

f: female; m: male; stage according to the classification of Durie and Salmon; ß2; beta2-microglobulin (mg/L); auto: high-dose melphalan (200mg/m²) and autologous PBSCT; allo: allogeneic PBSCT with reduced conditioning regimen (fludarabine 90mg/m², TBI); INF: maintenance therapy with interferon; Thal: maintenace therapy with thalidomide; CR: complete remission; PR: partial remission; MR: minimal response; PD: progressive disease; EFS: event-free-survival (days); †: death from infection; +: ongoing remission.

labeled with a fluorescence dye (HybProbes).[34] It has been shown for patients with ALL that IgH quantification assays can be transferred from one detection system to the other.[35,36]

To investigate whether both RQ-PCR techniques could be combined for IgH monitoring in MM we used one ASO primer, one consensus primer and an ASO Taqman probe together with the LightCycler system. The second goal of the study was to examine whether levels of residual tumor cells at given time points could identify patients with a high risk of relapse and whether changes in clonotypic cell numbers over time are predictive of relapse. Therefore, MRD levels of patients with MM following autologous and/or allogeneic peripheral blood stem cell transplantation (PBSCT) were correlated with clinical data to evaluate the prognostic value of quantitative molecular monitoring.

Design and Methods

Patients and samples

During routine diagnostic and procedures, 55 bone marrow (BM) and 107 peripheral blood (PB) samples were obtained from 11 patients with MM. The patients' characteristics are summarized in Table 1. Remission status was defined according to the EBMT criteria.[37] Informed consent was obtained from all patients and the study was performed according to the guidelines of the ethical committee of the University of Düsseldorf.

Mononuclear cells (MNC) from patients' samples were stored at -20°C. Cells from the myeloma cell line U266 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown in RPMI 1560 (10% FCS, 50 µg/mL penicillin, 50 µg/mL streptolysin) and either pelleted or serially diluted in 10-fold increments into normal PB MNC of healthy donors at dilutions ranging from one to 10^5 U266 cells in 10^7 normal MNC. CD 19^+ B cells were separated from MNC of healthy donors with MACS CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer´s recommendations. Genomic DNA was isolated using the QIAamp Blood Kit (Qiagen, Hilden, Germany) and total RNA was extracted using the RNeasy Mini Kit (Qiagen), again according to the manufacturer´s instructions. Concentrations of DNA and RNA were assessed by measurements of optical density (OD).

Amplification and sequencing of the patient-specific IgH

Total RNA from samples with high tumor load was reversely transcribed and a consensus PCR was performed using the Gene Amp RNA PCR core kit (Applied Biosystems, New Jersey, USA). In brief, 3 µL of total RNA were added to a mixture containing 1×PCR buffer, 5 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate (dNTP), 2.5 µM oligo(dT) primer, 1 U RNase inhibitor and 2.5 U MuLV RT. Reverse transcription was performed at 42°C for 15 minutes followed by 99°C for 5 minutes. Afterwards consensus PCR was performed with 1 µM FR1c-primer,[38] 1 µM JH_{1245}-, 1 µM JH_3- and 1 µM JH_6- primer[38] together with 1×PCR-buffer, 2 mM $MgCl_2$, 1 mM dNTP and 0.025 U AmpliTaq Polymerase (Applied Biosystems). Amplification conditions were as follows: 5 min preheating at 94°C, 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 59°C and 1.5 min elongation at 72°C, followed by a final extension of 8.5 min at 72°C. PCR products were analyzed by gel electrophoresis and excised from the gel for purification using the QiaQuick gel extraction kit (Qiagen). PCR products were then inserted in the pCR4-TOPO plasmid vector using the TOPO T/A Cloning Kit for Sequencing (Invitrogen, Paisley, UK) according to the manufacturer`s recommendations. Plasmid DNA

from 10 bacterial clones of each patient was purified using the QIAprep Miniprep Kit (Qiagen) and sequenced by a commercial supplier (SEQLAB, Göttingen) by automated cycle sequencing. A sequence repeated in at least three different clones was considered to be the patient-specific IgH sequence.

Design of ASO primers and ASO Taqman probes

The patient-specific IgH was compared with published V_H and J_H sequences of the V BASE directory (*www.mrc-cpe.cam.ac.uk*).

A sense ASO primer and an ASO Taqman probe were designed for the patient-specific complementary determining region (CDR) 3 using the PRIMER3 software (*www-genome.wi.mit.edu/genome_software/other/primer3.html.*) and advice from TIB Molbiol (Berlin, Germany). An appropriate consensus J_H primer (J_{H1245}, J_{H3} or J_{H6})[38] was chosen as the antisense primer. Probes labeled with FAM (6-carboxy-fluorescein) as reporter at the 5' end and the quencher TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end were obtained from TIB Molbiol (Berlin, Germany).

Development of plasmid standards for quantification

For quantification an external exogeneous standard with plasmids containing the patient-specific IgH sequence was used. DNA concentration of plasmids was calculated from three independent OD_{260} determinations. The copy number was calculated from the concentration, mean molecular weight of the nucleotides and the plasmid size. Then plasmid DNA was digested with 1 U Pst I (Biolabs, New England, USA) for 1 h at a concentration of 1×10^8 copies/μL, before the enzyme was inactivated at 95°C for 10 minutes. The plasmid solution was diluted in 6 ng/mL MS2 phage RNA (Roche, Mannheim, Germany) to a final concentration of 5×10^6 copies/μL and stored at -20°C.

To be sure that PCR efficiency did not differ between plasmid standards and genomic DNA of patients' samples, plasmids and DNA from U266 cells and one patient were serially diluted in DNA from healthy donors and analyzed by RQ-PCR.

Real-time PCR of patient-specific IgH and β-actin

The IgH-PCR reaction was carried out with the LightCycler system (Roche, Mannheim) in a final volume of 20 μL containing 1×LightCycler-FastStart Hybridisation Probes Reaction Mix (including FastStart Taq polymerase; Roche, Mannheim), dependent on target sequence, 0 to 7 mM $MgCl_2$, 0.5 mM ASO Primer, 0.5 mM J_H consensus primer (J_{H1245}, J_{H3} or J_{H6} dependent on target sequence), 200 nM ASO Taqman probe and 1 to 4 mg of sample DNA or 2 μL of diluted standards. Cycling conditions were as follows: one cycle of preincubation at 95°C for 10 min, 45 cycles of a denaturation step at 95°C for 0 sec and an amplification step at 60-67°C for 30 sec, one cycle of cooling at 40°C for 5 min. For fluorescence detection of Taqman probes the LightCycler software was set as follows: acquisition mode at the end of the amplification: *single*, channels: *F1/F2*, fluorimeter gains: *automated*.

For assessment of the linear amplification and the sensitivity of each patient-specific IgH PCR, 10-fold dilutions of the IgH plasmid in 660 ng normal cellular DNA corresponding to 10^5 cells were made and analyzed by RQ-PCR. To confirm the specificity of the assay each IgH RQ-PCR was tested with a panel of DNA from two healthy donors, two patients with MM, two patients with low-grade non-Hodgkin`s lymphoma and from sorted $CD19^+$ B cells from MNC of a healthy donor.

The β-actin PCR was run on the same instrument with the Taqman PCR Reagent Kit (Perkin Elmer, Weiterstadt) according to the manufacturer's instructions. Briefly, the PCR was run in a final volume of 20 μL containing 1×LightCycler-FastStart Hybridisation Probes Reaction Mix (Roche, Mannheim), 3.5 mM $MgCl_2$, 0.3 mM β-actin forward primer, 0.3 β-actin reverse primer, 200 nM β-actin Taqman probe and 1 to 4 μg of sample DNA or 2 μL of diluted standards. Cycling conditions were identical to those described for IgH PCR.

Analysis of patients' samples

IgH and β-actin copy number were determined for each sample and each sample was analyzed twice. A total of 5 measurements were made for negative samples. Three 10-fold dilutions of patient-specific IgH-plasmid, starting with 10^5 plasmid copies, and three 10-fold dilutions of human genomic DNA supplied by the Taqman PCR Reagent Kit (Perkin Elmer Biosystems, Weiterstadt) starting from 6×10^3 copies, were always co-amplified together with the patients' samples to create external standard curves for IgH and β-actin quantification. Mean IgH (1 allele) copy number was normalized by dividing by two the mean number of β-actin copies (2 alleles) multiplied by 100, thus obtaining an IgH/2β-actin ratio in percent, indicating the proportion of clonotypic cells in MNC from BM or PB.

Statistical analysis

Levels of clonotypic cells in BM were compared to those in PB using the Wilcoxon test. Correlation of results was analyzed with standard linear regression models. Standard curves were compared using multiple regression analysis with dummy coding. The coefficient of variation (CV) was calculated by dividing the standard deviation by the mean value. Kaplan-Meier plots were analyzed using the log rank test.

Table 2. ASO primers and ASO probes used for IgH RQ-PCR: for U266 and for patients #1-#11 individual sequences of the IgH VDJ region are shown using IUPAC codes. CDR3 sequences are in bold. The ASO primer (left) and ASO probe (right) for each patient are underlined.

U266	cystgtattactgtgcga**aaagtgaccctttttggagtgattattataactttgactactcgtacactttg**gacgtctggggcc
#1	acacggcystgtattactgtgcgagaa**cttttacatattactctgtttcgaagacttattataacgag**tactactttgactcctggggccagggga
#2	acacggctctgtattactgtgc**aaaagatcgtaactgg**gggggt**ggggaaggcttctttt**gactcctggggcc
#3	acacggccctgtattactgtgcgagaagcccccacccgggga**gggtggatc**actactttgactactggggccaggga
#4	cggcystgtattattactgtgcgcgaga**ttgg ggcattgtagtagacccagttgcgccc**gacttttggggccagggagccct
#5	agtcgaggacacggctgtttattactgtgcgagag**tacaaataccagctgctcttgattc**ctggggccaggga
#6	acacggccgtgtatttctgtgcgaga**gattatcatggcagcgcctggtaccctcgggcctttcaa**tactggggccaggga
#7	cacatattattgtgtacaca**ccccggggcagtggcaggccccc**gctgagtacttccagcactggg
#8	cggccgtatattactgtgc**cggcccggattattttgatactaa**gggccagggaaccct
#9	ctgtctattattgtgtgaga**caaaattgggggttcctaaaggaggtgggtacaacg**actactactactccggtctggacgtctggg
#10	ctgtttattactgtgtgaga**gacagagcgcttcgggacttagtccct**actactttgagcactggggccagggaacc
#11	tgtttattactgtgcgagaga**tgaagtgggagttgcgggg**gactactggggccagggaaccct

Results

Linear range, sensitivity, reproducibility and accuracyf or 11 MM patients and the myeloma cell line U266

A clone-specific IgH RQ-PCR was established using a sense ASO primer and an antisense J_H-consensus primer together with a patient-specific ASO Taqman probe (Table 2). Linear amplification conditions were found for each patient-specific IgH PCR with correlation coefficients of ≥ 0.98 for serial plasmid dilutions in a range from 10^6 to 10^1 copies. Slopes and intercepts of IgH standard curves were 3.4±0.2 and 40.7±3.6, respectively. The sensitivity of the assay was at least 10^{-4} (1 IgH copy in 10^4 cells). For some patients sensitivity was 10^{-5} (n=3) or even 10^{-6} (n=3). No positive signal was observed in any non-patient samples, reflecting the specificity of our assay.

Intra-assay and inter-assay variability were analyzed using 210 and 60 samples from PB and BM, respectively, which were obtained at different time points during the course of the disease and which contained different concentrations of clonotypic cells. We found a good reproducibility of the assay as reflected by a correlation coefficient of 0.99 and a CV of 0.27 (95% CI: 0.21-0.33) for intra-assay variability as well as a correlation coefficient of 0.99 and a CV of 0.38 (95% CI: 0.28-0.48) for inter-assay variability. To test the accuracy of the IgH RQ-PCR, U266 myeloma cells were diluted in normal PB MNC resulting in spiked samples with proportions of tumor cells ranging from 1% to 0.00001%. A close linear correlation (r = 0.99) was found between expected and measured values in samples with a tumor load from 1% to 0.01% indicating a high accuracy of the assay. In samples with a tumor load ≤ 0.001%, when less than 10 copies were present in a tube, the amount of tumor cells was overestimated (Figure 2).

To validate the use of plasmid standards, we analyzed dilutions of DNA and plasmid targets with the same cell equivalents for the U266 cell line and one patients' sample. Regression analysis of cell equivalents versus crossing points showed no significant differences (p = 0.8) indicating that the PCR efficiency did not differ between plasmid DNA and genomic DNA in our study (Figure 1).

Tumor load in peripheral blood and bone marrow

We performed an intra-individual quantification of myeloma cells in 30 PB and BM samples obtained at the same time. The samples were collected during the course of disease of 11 patients and included time points with different numbers of tumor cells. A negative PCR finding in PB, which occurred in 15 samples, was associated with a positive PCR result in two-thirds of the corresponding BM samples. In contrast, PCR negativity in five samples from BM was always accompanied by a negative PCR finding in PB. Tumor load in samples from PB was significantly (p < 0.001) lower than in samples from BM as reflected by median IgH/2β-actin ratios of 0.000023% (range: 2 to 0%) and 0.29% (range: 85 to 0%), respectively (Figure 3). The difference in IgH/2β-actin ratios in paired samples from BM and PB of individual patients varied by a factor of 2 to 650.

Amount of clonotypic cells before therapy, in remission and at the time of relapse

We report 11 cases of MRD from patients with MM following high-dose chemotherapy and autologous (n = 10) PBSCT and/or allogeneic (n = 2) PBSCT with reduced conditioning regimen. BM and PB samples from time points before and after PBSCT were compared. Six of eleven patients relapsed and samples were also examined at the time of progressive disease.

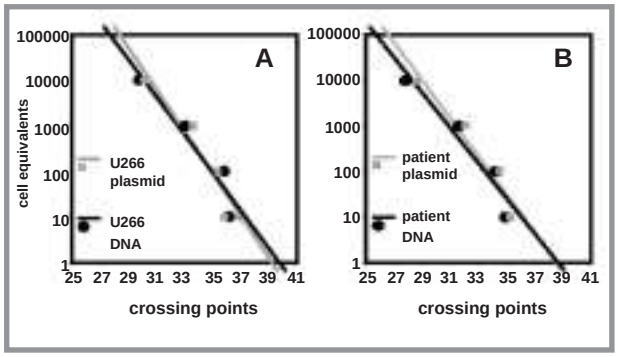

Figure 1. Plasmid DNA versus genomic DNA for standard curves. Plasmid DNA (■) and genomic DNA (●) of the U266 cell line (A) and a sample from patient #11 at the time of diagnosis (B) were diluted in 660 ng DNA of healthy donors. Tumor cell equivalents were plotted against crossing points showing equal amplification efficiency of both standards. Markers cover duplicate measurements of two independent experiments.

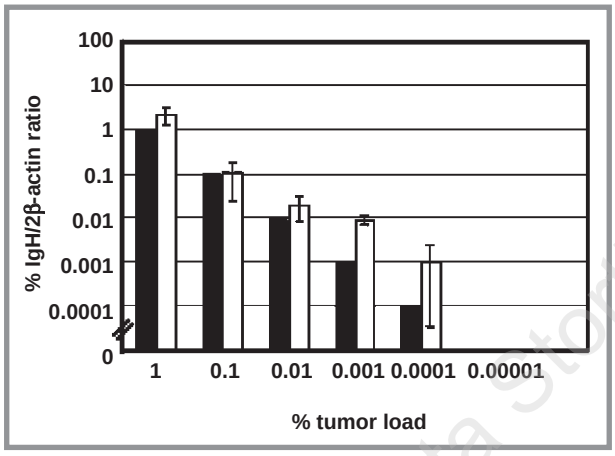

Figure 2. Evaluation of accuracy. The U266 cell line was diluted in MNC from healthy donors to produce samples that had a tumor cell content ranging from 1% to 0.00001%. Expected values and results of triplicates (1-0.1%)/quintuples (0.00001%) of 2 independent experiments are shown.

The intraindividual analysis of IgH/2β-actin ratios showed that the number of clonotypic cells was related to the state of disease. There was a significant reduction of IgH/2β-actin ratios (a median of 2 log) between pre-treatment values and those of best response in BM (median: 13% [range: 0.0001-100%] to 0.09% [range: 0-2.5%], $p = 0.003$ (Figure 4A). In PB the reduction of IgH/2β-actin ratios was even more pronounced (median: 0.03% [range: 0-1%] to 0% [range: 0-0.5%], $p = 0.02$ (Figure 4B). Relapse was associated with an increase of the median IgH/2β-actin ratio to 2% (range: 0.1-100%) in BM and to 0.4% (range: 0.1-2%) in PB (Figures 4A, B).

Analysis of the amount of clonotypic cells at given time points during therapy revealed that IgH/2β-actin ratios from samples collected at the time after induc-

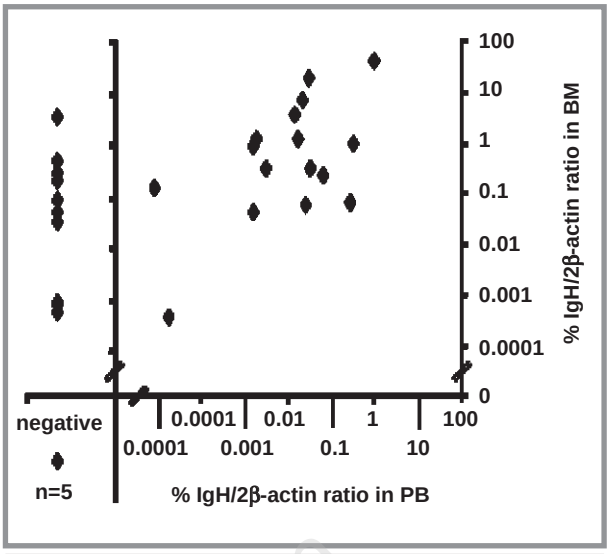

Figure 3. Differences in tumor load in BM and PB: IgH/2β-actin ratios of 30 paired BM-PB samples with low and high tumor load taken at the same time are shown. IgH/2β-actin ratios of PB and BM samples are plotted on the x-axis and y-axis, respectively.

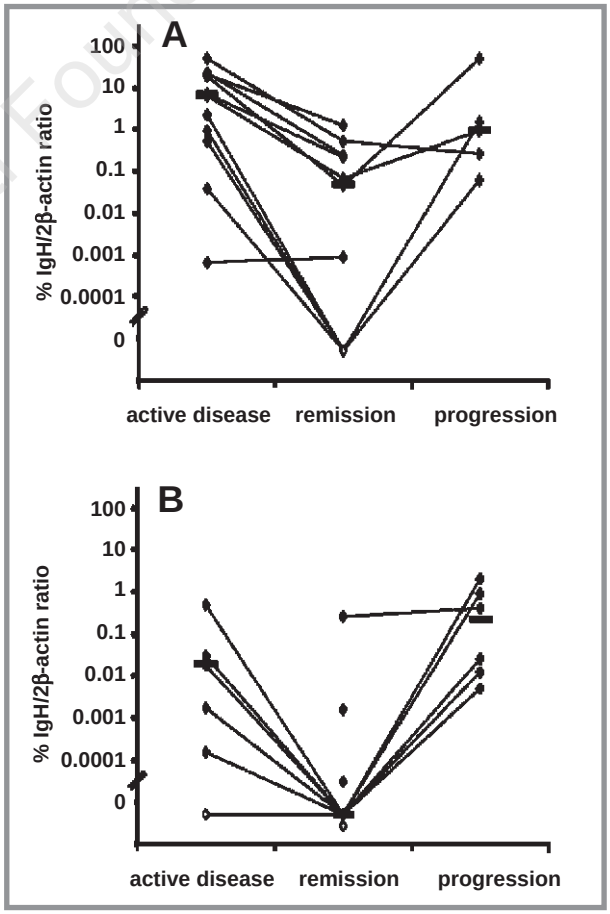

Figure 4. Association with state of disease. IgH/2β-actin ratios in 11 MM patients at the time of active disease (time of diagnosis, during induction therapy), remission (best response during remission) and progressive disease in BM (A) and PB (B). Bars indicate median IgH/2β-actin levels. Open circles show negative PCR results.

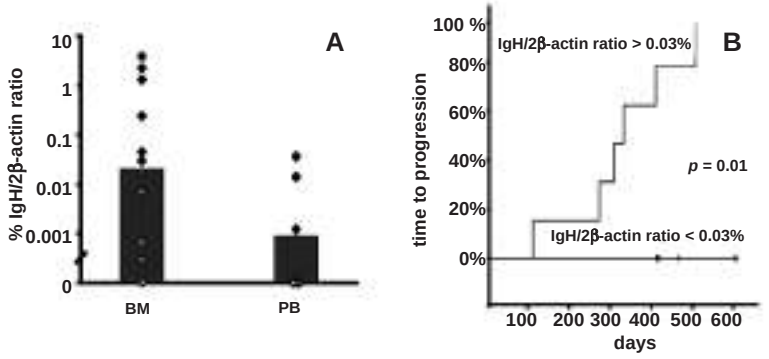

Figure 5. Level of clonotypic cells prior to PBSCT. A. IgH/2β-actin ratios in BM and PB samples from 11 patients at the time after induction therapy and before PBSCT. Closed diamonds represent values from patients with relapse following PBSCT (median time to progression 10.8 months), open diamonds represent values from patients with ongoing remission (median observation time 17.1 months). Gray boxes indicate a potential prognostic cut-off level. B. MRD level in BM before transplantation identifies two groups of patients with different risks of relapse. Time-to-progression after transplantation differed significantly between patients with a pre-transplantation IgH/2β-actin ratio in BM of < 0.03% and patients with higher MRD levels.

tion chemotherapy but before PBSCT were associated with the probability of relapse. At this time point two groups of patients could be identified using a threshold level of 0.03% for IgH/2β-actin ratio in BM. Time-to-progression in these two groups differed significantly ($p < 0.01$) after a median observation time of 13 months (Figure 5B). IgH/2β-actin ratios of more than 0.03% in BM samples or above 0.001% in PB samples were found in all patients who relapsed (median time-to-progression: 10.8 months). IgH/2β-actin ratios of all patients with ongoing remissions after PBSCT (median follow-up: 17.1 months) were below these levels (Figure 5A). Whereas the amount of clonotypic cells before transplantation was associated with the probability of relapse, no association could be found with paraprotein levels, bone marrow infiltration, β2-microglobulin and lactate dehydrogenase levels or the presence of cytogenetic abnormalities. Two case reports exemplify this finding. Two male patients, 41 and 45 years old, with stage III A multiple myeloma of IgGκ subtype and normal BM karyotype were treated with induction chemotherapy followed by high-dose melphalan chemotherapy with autologous SCT and maintenance therapy with interferon α.

One patient, who had 80% BM infiltration and a β2-microglobulin of 3.0 mg/L, achieved a complete remission with negative immunofixation but relapsed 4 months after transplantation whereas the other patient, who had 60% BM infiltration and a β2-microglobulin of 2.3 mg/dL, achieved a minimal response with a less than 50% reduction of monoclonal protein which lasted for more than 423 days. Whereas conventional prognostic parameters, such as cytogenetic abnormalities or β2-microglobulin level, were not predictive of the clinical outcome of these identically treated patients, the IgH/2β-actin ratios of 0.47% and of 0.0013% in BM at the time before transplantation discriminated very well between the patient who went on to have an early relapse and the other patient who had long-lasting remission, respectively.

Sequential molecular monitoring of patients following therapy

We report 11 cases of sequential monitoring of MRD in PB from patients with MM following PBSCT. A median of 9 samples (range: 2-17) per patient were analyzed. Ongoing remission was observed in 5 patients, with constantly negative IgH RQ-PCR in three patients and a persistent low level of residual tumor cells (median IgH/2β-actin ratio: 0.0003%) in the other two. On the other hand, there were six patients who relapsed (Figure 6 A-F). In patients A-D relapse was associated with an at least 10-fold increase of the IgH/2β-actin ratio 3 months (median, range: 0.5-6) before the onset of relapse. Two patients (A,C) were in complete remission with a negative immunofixation result at the time when a rise of the IgH/2β-actin ratio was observed. Another two of the relapsed patients (B,D) had partial remissions without significant changes in the concentration of the monoclonal protein measured by electrophoresis at the time when an increase of IgH/2β-actin ratio was observed. In patients E and F the rise of IgH/2β-actin ratio in PB was found simultaneously with the conversion of negative to positive immunofixation and the beginning of a rising M gradient, respectively.

Discussion

In patients with MM, quantitative IgH PCR permits the measurement of residual clonotypic cells in samples from PB and BM before and after therapy. At present, different PCR methods are proposed for quantitative monitoring of MRD in patients with MM, although the prognostic significance of changes in the amount of residual tumor cells is still not clear.

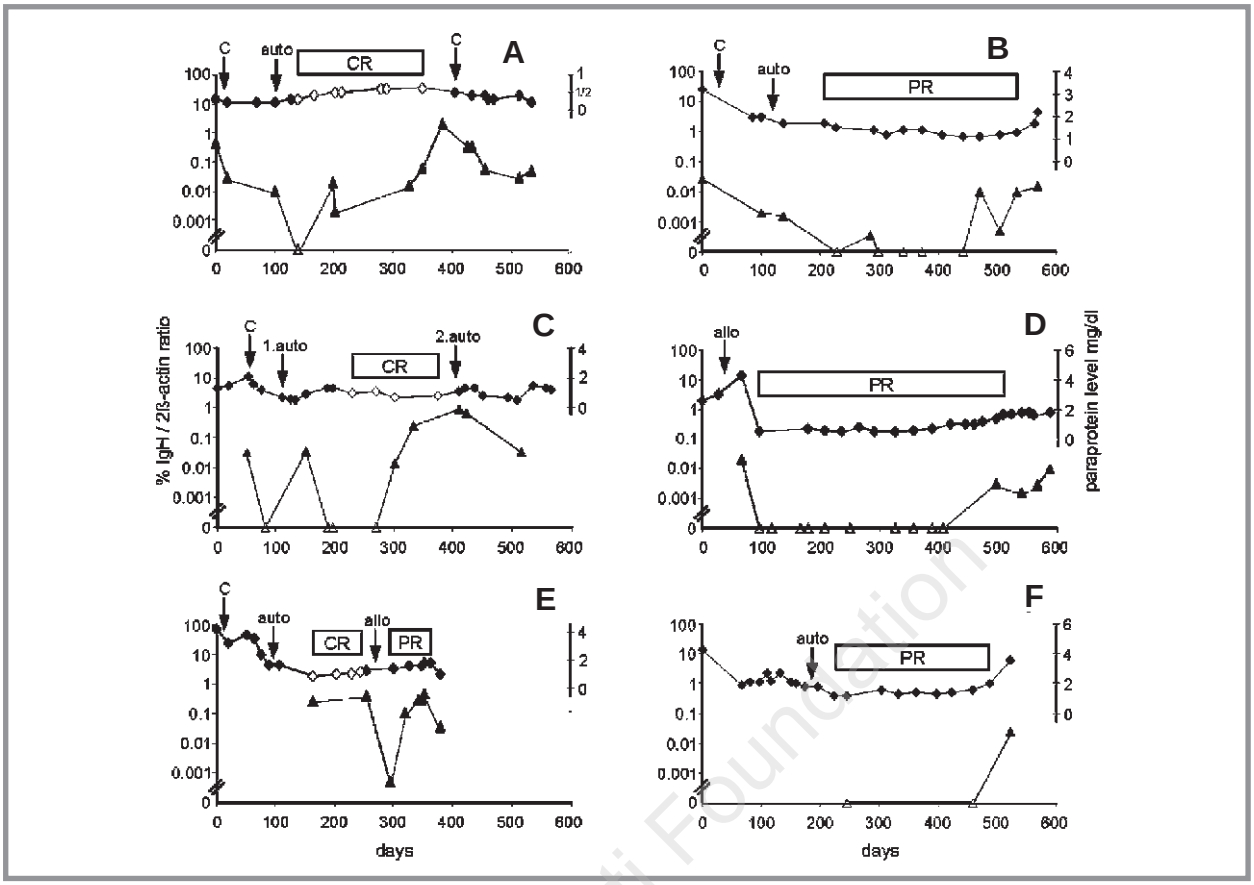

Figure 6. Sequential monitoring of MRD in PB. Changes of clonotypic cell numbers in PB are shown for 6 patients (A-F = #6-11) with relapse after PBSCT. Paraprotein levels (g/dL) measured by electrophoresis are represented by diamonds in the upper line by the right y-axis, IgH/2β-actin ratios are represented by triangles in the lower line by the left y-axis. Open diamonds indicate no M gradient in the electrophoresis and a negative immunofixation, open triangles show negative PCR results. Start of chemotherapy (= C) and time of high-dose therapy (= auto) and/or allogeneic PBSCT (= allo) are shown by arrows. The duration of remissions is indicated by rectangles (CR: complete remission; PR: partial remission).

In this study, we provide further evidence for the prognostic relevance of quantitative MRD monitoring in MM. We show that results of sequential molecular monitoring of MRD following high-dose therapy were able to predict a relapse in two-thirds of the patients. Furthermore, our study suggests the existence of a cut-off level for residual tumor cells in samples obtained prior to PBSCT with prognostic impact for the probability of relapse. We also show that the amount of residual tumor cells is associated with the state of disease and that the extent of MRD in samples from PB and BM differs significantly in patients with MM. Moreover, we established a method for quantification of MRD in patients with MM combining two alternative RQ-PCR systems. The use of TaqMan chemistry together with LightCycler technology makes performance of patient-specific IgH RQ-PCR a reliable technique.

In our study pre-transplantation IgH/2β-actin values in BM could discriminate two different prognostic groups of patients. This suggests that, as shown for patients with ALL,[16] there might be a pre-transplantation cut-off level of residual tumor cells in samples from BM and PB that allows the identification of patients with MM at a high risk of relapse. The observation that conventional prognostic parameters did not correlate with IgH/2β-actin ratios highlights the usefulness of IgH RQ-PCR as an independent molecular parameter, which provides additional prognostic information for individual patients. Moreover, our findings provide further evidence of the prognostic value of pre-transplantation levels of tumor cells[39,40] and the need to improve induction therapy. Similar results were reported in a study using limiting dilution IgH PCR instead of RQ-PCR. Bakkus *et al.*[27] showed, for the first time, that there is a threshold of post-transplantation tumor load in BM which has prognostic significance regarding the progression-free survival of patients with MM. Before MRD testing can be used in the management of patients with MM, as it is already in the management of patients with leukemia, data from different groups and with larger numbers of patients are necessary. Then, meas-

urement of IgH/2β-actin ratios could lead to treatment stratification on a molecular level in patients with MM.

Besides the prognostic value of residual tumor cells at a given time point, our study highlights the role of sequential quantitative monitoring of MRD. To date, published results on changes of the amount of tumor cells during different kinds of therapy of patients with MM are limited.[24,31,41,42] Looking at BM in 11 patients, we observed a significant, 2-log reduction of clonotypic cells between the time of active disease and that of best response after PBSCT. This is in accordance with findings of other groups[24,31,41] who reported a similar reduction in the number of tumor cells in BM. During relapse after PBSCT we found an increase of IgH/2β-actin ratio, which was also described by Lipinsky *et al.*[25] Looking at the amount of clonotypic cells in PB, different groups have produced conflicting data. In line with Cremer *et al.*[24] and in contrast to others,[41,43] we observed a significant reduction of clonotypic cells after high-dose therapy in patients who achieved remission, and a subsequent rise in patients at the time of progressive disease. Most importantly, a continuous increase of IgH/2β-actin ratios of more than 1 log preceded disease progression in 66% of relapsing patients 3 months before other examinations indicated relapse. This suggests that quantitative IgH monitoring in PB is of value in predicting relapse in MM after autologous PBSCT. Patients with rising IgH/2β-actin ratios after high-dose chemotherapy could be candidates for dose-escalated maintenance therapy or for treatment with novel drugs, such as Revimid or Velcade. Our findings, using RQ-PCR to analyze a median of 9 PB samples from each patient, are in line with those of Cremer *et al.*,[24] who used limiting dilution PCR and showed results from PB samples at three time points after transplantation. Two other studies[41,43] also reported results from sequential MRD monitoring in patients with MM following high-dose chemotherapy and autologous PBSCT, with only one study providing data on samples from two patients at the time of disease progression. Furthermore, our findings demonstrate the prognostic importance of quantitative molecular IgH monitoring not only for patients after allogeneic PBSCT[3] but also for patients after autologous PBSCT, who represent the majority of all patients with MM.

With growing evidence of the prognostic value of monitoring of MRD in MM, methodological questions arise. There are different methods for quantifying clonotypic cells in B-cell malignancies by IgH PCR. ASO or consensus probes provide equal specificity and sensitivity[29-32] when stringent criteria for the effectiveness of RQ-PCR are applied.[29] It has been shown that only those patients who have less than three total mutations in both probe and primer binding sites fulfill the conditions for an optimal RQ-PCR.[29,32] In our study the ASO primer and probe assured 100% homology to the target sequence. The use of a consensus reverse primer, which is identical to the plasmid DNA used as the external standard, but which is potentially less perfectly matched to the DNA of the myeloma clone, could theoretically cause a bias in PCR amplification efficiency. Our data comparing standard curves from cloned DNA with standard curves made from DNA of heavily infiltrated BM showed that using one antisense consensus primer did not result in a significantly different PCR efficiency. Similiar results were described by Gerard *et al.*[44]

Taqman probes are more suitable than HybProbes for the design of ASO probes, because of the small size of the patient-specific CDR3. We show that Taqman probes can be used with the LightCycler system to quantify IgH copy numbers in patients with MM, as has been shown for patients with ALL.[35,36] The sensitivity, specificity, accuracy and reproducibility of our assay were comparable to those of other assays by groups using hybridization probes with the LightCycler system or a hydrolization probe with the ABI Prism SDS.[21,29-32,45] Thus, for quantitative IgH monitoring in patients with MM the application of Taqman probes is independent of the fluorescence detection system used. The possibility of using different equipment facilitates the realization of multicenter studies, which are required for further evaluation of the clinical relevance of IgH monitoring in patients with MM.

Another important question, alongside the methodological considerations, is whether samples from PB or BM should be used for monitoring. While it is very easy to obtain blood samples on a regular basis, allowing monthly monitoring, we found a lower number of clonotypic cells in samples from PB than in those from BM. The difference in IgH/2β-actin ratios between paired BM and PB samples varied over a range of 3 logs. It is not clear whether this variation was due to the heterogeneous BM infiltration[31] in comparison to the homogeneous distribution of tumor cells in PB. Another explanation for this variation could be the variable contamination of BM samples with different volumes of PB. Pragmatically, we suggest that both BM and PB are examined with RQ-PCR.

In conclusion, this study has shown that quantitative molecular monitoring in patients with MM following PBSCT is of prognostic value and is, therefore, helpful for guiding therapeutic interventions. Further studies are necessary to confirm these results in larger groups of patients.

RF planned, performed and analyzed the experiments reported. He wrote and revised the manuscript. MA, CA and MK performed experiments providing several data presented in the publication. MA created Figure 2 and Table 2. CA created Figures 1 and 3. MK created Fig-

ures 4-6. GK, AH and AB were significantly involved in patient recruitment and correlation of clinical data with experimental findings. GK created Table 1. US was involved in the conception and design of the study and substantially contributed to the development of the methodology. UR was involved in the conception and design of the study and provided meticlous technical advice. SK was substantially involved in the analysis and interpretation of the data. RH was significantly involved in the induction and conception of the study. RK was significantly involved in the induction and conception of the study and contributed substantially to the development of the methodology. All the authors critically revised the manuscript and approved the submitted manuscript. The authors reported no potential conflicts of interest.

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