

ORIGINAL ARTICLE

The evaluation of minimal residual disease in multiple myeloma by fluorescent molecular beacons in real time PCR of IgH gene rearrangements and correlation with flow cytometry

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Summary

Purpose: Multiple myeloma (MM) patients relapse after a period of time despite longer disease-free survival due to novel treatment options. In this study we aimed to assess the value of real-time polymerase chain reaction (RT-PCR) for detecting the immunoglobulin heavy chain (IgH) gene rearrangement using allele-specific molecular beacons as fluorescence probes to quantify minimal residual disease (MRD) and also to correlate post-treatment flow cytometric detection of plasma cells' (PCs) expression of CD19, CD38, CD45, CD56 and CD138 in MM.

Methods: After diagnosis of 17 MM patients, the CDR1, CDR2 and CDR3 regions of the IgH gene were analysed and sequenced to identify IgH's clonal nature. Unique sequences of the clonal IgH rearrangement were used to design specific molecular beacon probes for each MM patient. Examined were also the co-expression of CD19, CD38, CD45, CD56,

and CD138 molecules in bone marrow aspirates of patients with MM by flow cytometry.

Results: Detection of MRD was positive in 13 (76%) of 17 patients by RT-PCR. The infiltration ratio was significantly correlated with CD138 expression ($p=0.009$). Significant correlation was also found between RT-PCR detection of MRD and CD138 expression ($p=0.006$). Nevertheless, no correlation was observed among other surface antigens (CD38, CD45, CD56).

Conclusion: Our results indicated that RT-PCR with specific molecular beacons provide a feasible, accurate and reproducible method for the determination of MRD in MM. Flow cytometry detection of CD138 expression may be used as a disease marker in addition to RT-PCR.

Key words: CD138, flow cytometry, minimal residual disease, multiple myeloma, real-time PCR

Introduction

The response rates in MM have been improved with high-dose chemotherapy followed by autologous stem cell transplantation and newer chemotherapeutic agents such as thalidomide, lenalidomide, and bortezomib [1-3]. After intensive treatment, most of the MM patients show persistent residual disease [4].

The presence of normal PCs, hemodilution of bone marrow aspirate and changes of the immunophenotype are important considerations in MRD assessment in MM patients [5]. Four cytogenetic subcategories are recognized in MM. Hypodiploid,

pseudodiploid, hyperdiploid, and near-tetraploid MM. The hypodiploid, pseudodiploid, and near-tetraploid karyotypes can be referred to as the nonhyperdiploid MM. IgH translocations are seen in 60% of the patients. The recurrent IgH translocations are highly associated with nonhyperdiploid variant MM [6]. No specific molecular marker has yet been identified to characterize the clonality of the cellular population in MM, and information is typically obtained by identification of a particular IgH gene rearrangement [7]. RT-PCR of the IgH gene in the majority of MM patients in complete remission after autologous stem cell transplantation identifies a single clonal popula-

tion [8-12].

Sensitive and robust assays are, therefore, required to quantify MRD following therapy, to monitor the effects of the novel treatment strategies and to identify patients at risk of disease progression and who may require additional treatment [5].

CD38, CD138, CD45, CD19, CD56, CD20, CD52 and CD117 are flow cytometric parameters used as predictive factors in different studies for various conditions in MM. CD38, CD138, and/or CD45 have been used for identifying PCs in a milieu of hematopoietic cells [12,13]. CD19 and CD56 have been used to differentiate neoplastic from normal PCs [14-16], and CD20, CD52 and CD117 have been used to identify the benefit of antigen-directed therapy [17-20].

In this study, we aimed to evaluate RT-PCR for the IgH gene using allele-specific molecular beacons as fluorescence probes to quantify MRD and also to correlate flow cytometric detection of PCs in MM patients during follow-up following treatment with high-dose or standard chemotherapy.

Methods

Patients included in this study were diagnosed with MM using standard clinico-laboratory criteria [21] and were subsequently included in a treatment protocol involving high-dose chemotherapy with autologous stem cell transplantation after first-line standard chemotherapy. The CDR1, CDR2 and CDR3 regions of the IgH gene were analysed and sequenced to identify its clonal nature. Sequenced genomic DNA was isolated from bone marrow. RT-PCR for the CDR1, CDR2 and CDR3 regions of the IgH gene was previously described by Aubin et al. [22]. Unique sequences of the clonal IgH rearrangement were used to design specific molecular beacon probes for each MM patient. The IgH primers employed were Fr1c 5'-AGGTGCAGCTGG/CA/TGG/CAGT CA/G/TGG-3', Ca2 5'-AA CTGCTGAG-GAGACGGTGACC-3'. The molecular beacon sequences were 5'-CCCCCTTGGCCCCAGACGTCCGGGGG-3' and 5'-

CCGGGGGCAGCTGGTACTACCACAATAGTCCCCGG-3' for 17 patients, respectively. The b-globin primers were globin-F 5'-ACACAACTGTGTTCAC TAGC-3' and globin-R 5'-CAACTCATCCACGTTCACC-3' and the molecular beacon was 5'-CGCGCG-GAGAAGTCTGCCGTTACTGCCCTGCGCGCG-3' [23]. All molecular beacons were labelled at the 5' end with 6-carboxy-fluorescein (FAM) and at 3' end with Dabcyl.

Real time quantitative PCR

Duplicate RT-PCR amplifications were carried out in a Light CyclerTM (Roche Biochemicals, Mannheim, Germany) using Fast Start Light CyclerTM DNAMaster,

containing Taq-polymerase, reaction buffer and dNTPs (Roche Biochemicals, Mannheim, Germany). All reactions were performed in 10 µl volumes and fluorescence quantification was calculated with the aid of built-in Light Cycler software, version 3.01 (Roche Biochemicals, Mannheim, Germany). For RT-PCR of IgH 1.5 ml of the corresponding molecular beacon were used.

Optimal fluorescence acquisition was determined experimentally for each probe, which in turn required modification of the cycling programme. For RT-PCR of b-globin, 1 ml of each primer (globin-F and globin-R) and 0.5 ml of the corresponding molecular beacon were used at 4 mM MgCl₂ containing material. Cycling conditions were 45 cycles of denaturation (94 °C/0 sec), annealing and fluorescence acquisition (60 °C/10 sec) and elongation (72 °C/12 sec). Standard curves for quantification were prepared by making serial 10-fold dilutions of the plasmid containing the rearranged clonal IgH from each patient and the purified b-globin fragment of PCR product.

Flow cytometry

We also examined the co-expression of the CD19, CD38, CD45, CD56 and CD138 molecules in cells of bone marrow aspirates in MM patients by flow cytometry. We used a two-step acquisition procedure in which up to 2x10⁶ cells were acquired through a specific live-gate drawn on SSC/CD38⁺⁺⁺/CD138⁺ cells to increase the level of sensitivity of the technique. In all cases, a FL1/FL2/FL3 isotype-matched negative control CD38 for antigen-positive cells was used to specifically evaluate the autofluorescence level of the PCs. Paint-A-Gate software (Becton Dickinson, San Jose, CA, USA) was used according to well-established methods for data analysis [24]. The main variable evaluated in this study was the percentage of phenotypic package program was used in the statistical analysis of data. Results were evaluated by an ally aberrant MM-PCs in the whole bone marrow.

Statistics

The SPSS, v.18.0 p expert at the Department of Biostatistics. Categorical measurements were summarized as numbers and percentages, whereas numeric measurements were given as average and standard deviation. The chi-square test was used in the comparison of categorical measurements between different groups. The level of statistical significance was set at 0.05 in all tests.

Results

Active disease was defined as PCs infiltration ratio >10% in bone marrow and also in patients labeled by CD38 and CD138 by flow cytometry. The patient median age was 55 years (range 40-70). Fourteen patients (82%) were male and 3 (18%) female. The detection of MRD was positive

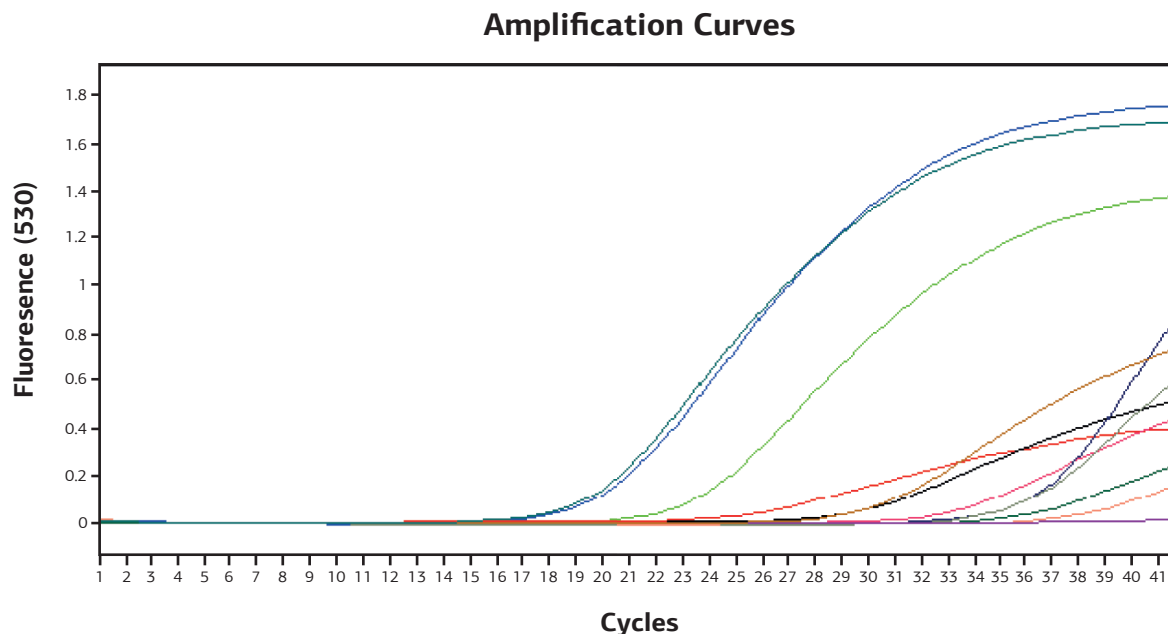


Figure 1. RT-PCR amplification curves of the patients. PCR assay of IgG H using molecular beacons. Different colors show the different patients. Standard curves for quantification were prepared by making serial 10-fold dilutions of the plasmid containing the rearranged clonal IgG H from each patient and the purified b-globin fragment of the PCR product.

in 13 (76%) patients by RT-PCR (Figure 1). The infiltration ratio was highly correlated with CD138 expression ($p=0.009$) and RT-PCR detection of PCs ($p=0.006$), and significant correlation was found between RT-PCR detection and CD138 expression ($p=0.006$). No correlation was found between other surface antigens (CD38, CD45, CD56; Table 1).

Discussion

MM patients relapse after a period of time despite longer disease-free periods and overall survival [25]. Novel chemotherapeutic agents and high-dose chemotherapy with autologous stem cell transplantation have improved response rates [5]. MM patients have a high rate of complete remission following these treatment approaches. Although median response duration usually does not exceed 3 years [26], MRD detection can show the effectiveness of treatment and can be a predictive factor in deciding treatment intensification. Relapse risk can be identified by MRD detection and treatment can be realized by the guidance of MRD, by identifying patients who are likely to relapse and who may therefore benefit from more intensive therapy [25].

Persistence of residual tumor cells is responsible for disease recurrence [1,26]. Disease recurrence still remains the leading cause of death [27]. Several studies have shown that residual disease above 0.01% is clinically relevant in MM [12,26,27].

The patchy nature of residual PCs and hemodilution of bone marrow aspirates decrease the quantification of PCs in bone marrow cellularity. Hence, multiparametric flow cytometry (MPFC) for MRD analysis is less sensitive than allele-specific oligonucleotide RT-PCR. False-negative results are possible when analyzing MRD with flow cytometry more often than allele-specific oligonucleotide RT-PCR [5]. In this study we analyzed the impact of RT-PCR in identifying MRD and also examined the co-expression of CD19, CD38, CD45, CD56 and CD138 molecules in cells of bone marrow aspirates in MM patients with flow cytometry.

We used fluorescent molecular beacons in RT-PCR. Molecular beacons are hairpin-shaped oligonucleotide probes that identify the presence of specific nucleic acids in homogeneous solutions. When they bind to their targets in the PCR products they undergo a conformational reorganization that restores the fluorescence of an internally quenched fluorophore [9-11]. These probes are designed in such a way that a loop portion of the molecule is complementary in sequence to the target nucleic acid molecule. The stem is formed by annealing of the self complementary arms at the 5'- and 3'-ends of the probe. These arms are typically guanine+cytosine rich to increase the bond strength in the short sequence stretch of the stem [4].

In the present study the detection of the MRD was positive in 13 patients by RT-PCR with fluores-

Table 1. Descriptive characteristics of the patients

Patient no.	Age, years/ Sex	Stage	LDH	IgG	Post tx* BMI (%)	Follow up period/mos (after tx)	CD19	CD38	CD45	CD56	CD138
1	55/M	IIIB	366	1339	<5	1	3.2	14.0	78.0	7.0	4.2
2	56/M	IIIB	319	1259	<5	6	1.4	64.2	99.8	0.5	8.5
3	70/F	IIIA	599	3255	<5	1	5.5	15.4	90.0	25.0	7.9
4	65/M	IIIA	425	1246	<5	48	2.2	60.3	100	3.0	10.8
5	60/M	IIIA	440	1458	<5	3	1.3	76.3	99.4	6.7	48.0
6	48/M	IIIA	371	2361	<5	31	6.0	91.0	90.0	7.0	4.0
7	70/M	IIIA	293	838	<5	26	15.0	23.0	90.0	6.5	1.8
8	58/M	IIIA	364	1141	<5	4	2.0	88.0	99.6	11.4	8.0
9	52/M	IIIA	311	8952	<5	19	2.0	83.0	99.9	14.0	12.0
10	60/F	IIIA	328	726	<5	59	0.2	75.0	99.5	21.0	3.1
11	55/F	IIIA	450	1650	<5	16	3.5	71.0	99.0	10.0	3.4
12	46/M	IIIA	345	1590	<5	18	20	45.0	90.0	2.1	1.8
13	40/M	IIIA	676	2717	<5	14	2.1	59.0	36.0	19.0	10.4
14	46/M	IIIA	500	1343	<5	6	46.9	58.3	82.0	15.4	6.3
15	48/M	IIIA	970	5200	<5	1	0.7	89.0	80.0	26.6	21.0
16	55/M	IIIA	399	1516	<5	48	2.0	88.0	46.0	11.4	8.0
17	50/M	IIIA	444	1420	<5	3	0.4	98.3	99.0	6.8	13.4

tx*: bone marrow transplantation, M: male, F: female, mos: months
LDH: lactate dehydrogenase, IgG: immunoglobulin, BMI: bone marrow infiltration index

cent molecular beacons. The infiltration ratio was correlated with CD138 expression ($p=0.009$) and RT-PCR detection of PCs ($p=0.006$) and also significant correlation was found between RT-PCR detection and CD138 expression ($p=0.006$).

Several studies were performed for detection of MRD in MM [4,5,26-28]. Bakkus et al. analyzed 64 patients for MRD detection by using semi-quantitative allelic specific oligonucleotide (ASO)-PCR. This study identified a threshold of the post high-dose chemotherapy tumor load with the prognostic value for progression free survival (PFS) in MM [27]. Fenk et al. analyzed 11 patients before peripheral blood stem cell transplantation by using real-time quantitative ASO-PCR and reported that following stem cell transplantation a significant reduction of clonotypic cells was observed in the bone marrow and peripheral blood samples of all 11 patients comparing the pretreatment values with those of best response (median 13-0.09% and 0.03-0%, respectively). Martínez-López et al. analyzed 2 patients and indicated that RT-PCR with specific molecular beacons provides a feasible method for the detection of MRD in MM [4]. We used molecular beacons in RT-PCR for detection of MRD in 17 MM patients and,

like Martínez-López et al. [4], we found that fluorescent molecular beacons in RT-PCR of IgH gene rearrangements is a feasible and accurate method for evaluation of MRD in MM in larger patient groups.

We also aimed to find a possible correlation with MFCM in this study. RT-PCR is a more sensitive method than MFCM, whereas MFCM is a simpler and faster method than RT-PCR [25]. Gupta et al. have reported on the MFCM method for the detection of MRD in 124 subjects (107 with MM, 11 with Hodgkin's lymphoma and 6 with allogeneic stem cell donors). The immunophenotypes of normal and reactive PCs were similar and different from those of neoplastic PCs with respect to CD19, CD45, CD56, CD 52, CD20 and CD117 in their study, and MRD was detected in all samples by using MPFC [5]. Sarasquete et al. [26] primarily compared MRD detection by using MFCM and allelic-specific oligonucleotide-real time quantitative PCR (ASO-RQ-PCR) in their report. Thirty-two patients with complete response following transplantation were analyzed in their study. MRD evaluation by ASO-RQ-PCR was found to be slightly more sensitive and specific than MFCM. MFCM yielded similar prognostic information with the advantage of being a more sim-

ple and faster method in their report. We analyzed the IgH gene of 17 patients with both techniques (MFCM and RT-PCR) using allele-specific molecular beacons; we found only CD138 co-expression in correlation with RT-PCR but not with CD38, CD45 and CD56.

RT-PCR and MFCM can be complementary techniques in MRD evaluation for MM. Both techniques show that decrease in the bone marrow load below 1 malignant cell per 10 000 bone marrow cells could be used as a target for the definition of a molecular/immunophenotypic complete remission [27]. The limitation of MFCM is the patchy infiltration and hemodilution of bone marrow, making it less sensitive technique than RT-PCR [4].

In conclusion, MRD in MM is a predictive and prognostic factor and an indicator of treatment response. Detection of MRD in MM by RT-PCR of the

IgH gene using allele specific molecular beacons is a reliable method, but needs many more patients to be evaluated in prospective clinical trials. CD138 co-expression was determined in correlation with RT-PCR by using allele-specific molecular beacons. In this study RT-PCR with specific molecular beacons was shown to be a feasible, accurate and reproducible method for the determination of MRD in MM. Only CD138 expression by MFCM may be used as a disease marker in addition to RT-PCR detection.

Contributions

IOK designed the study, analyzed the data, wrote the paper and gave final approval of the paper. BBD analyzed the data, wrote the paper and gave final approval of the paper. CUA analyzed the data, wrote the paper.

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