Detection of tumor-specific marker for minimal residual disease in multiple myeloma patients

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Aims. Multiple myeloma (MM), the second most common hematological cancer, is a lymphoproliferative disease of terminally differentiated B lymphocytes characterized by expansion of monoclonal plasma cells. With the introduction of new drugs, MM has become a hard-to-treat disease. The aim of treatment is clinical remission and eradication of clinical manifestations but most MM patients eventually relapse. For this reason, more accurate monitoring of remission and relapse using molecular biology techniques is at the center of attention.

Methods. For monitoring, we used allele-specific PCR and quantitative real-time PCR based on specific detection of VDJ immunoglobulin heavy chain gene rearrangement of clonal cells for monitoring. The hypervariable region of IgH rearrangement is used for detection of minimal residual disease (MRD) in MM as this sequence is used for allele-specific primers and probe design. This technique is a complementary tool for flow cytometry in MRD detection; however, it has not been established in the Czech Republic so far.

Results. Qualitative and quantitative MRD detection was performed in 50% (5/10) patients and qualitative MRD detection in another 3 oligoclonal patients.

Conclusions. Next to flow cytometry, detection of MRD by qPCR is a viable option and has been introduced in the Czech Republic.

Key words: multiple myeloma, minimal residual disease, tumor-specific marker, ASO PCR, RQ-PCR, IgH gene rearrangement

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INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy that is ranked among B lymphoproliferative neoplasias by the World Health Organization¹. MM is a complex disease characterized by accumulation of clonal malignant plasma cells (PC) in the bone marrow (BM) together with production of monoclonal immunoglobulins or light/heavy chains, resulting in clinical manifestation of the disease. Osteolysis, hypercalcemia, anemia, immune system impairment and renal insufficiency are among the most common clinical manifestations of MM (ref.^{2,3}).

With the introduction of new drugs and use of autologous stem cell transplantation, MM is slowly turning into a chronic disease. The aim of the treatment is hematological remission and eradication of clinical manifestations. Nevertheless, most MM patients eventually relapse³. This implies that not all clonogenic malignant cells had been killed and that the residue of malignant cells persisting even after treatment contributes to recurrence of the disease.

For this reason, more accurate monitoring of remission and relapse by molecular biology techniques is important. One of these techniques is allele-specific (ASO) PCR

and a real-time quantitative PCR (RQ-PCR) based on analysis of junctional regions of rearranged immunoglobulin heavy chain (IGH) gene⁴. The hypervariable region of IgH rearrangement is used as a tumor marker for detection of minimal residual disease in MM. Determination of such marker and its sequence analysis further allows for allele-specific (ASO) primers and probe design⁵.

MRD detection using PCR has major advantages because of its sensitivity, accuracy, reproducibility, need of small amount of DNA and widespread and irreplaceable use in retrospective studies. On the other hand, PCR methods are more complex, expensive, take more time and allow detection of only one clone that was present at the time of diagnosis⁶. However, detection of tumor marker by PCR has a wide application for clinical evaluation of patients, for early relapse detection or for quantification of tumor contamination in healthy hematopoietic cells for autologous transplantation⁷.

The use of flow cytometry (FC) for MRD detection appears to have prognostic significance as well^{6,7}. One current approach is MRD detection using an 8-color polychromatic FC. This technique is also able to differentiate the expression of immunoglobulin light chain (IgL) κ or λ (ref.^{8,9}). MRD detection via FC is applicable in

approximately 90% of MM patients, which is important for routine practice⁶. The significance of FC use in MRD detection was shown in the large studies of Paiva et al. In these studies, patient treatment response was evaluated via immunofixation, serum free light chain, multiparameter FC immunophenotyping and FC together with assessment of high-risk cytogenetics^{10,11}.

Unfortunately, there has never been a comparative study of PCR vs. FC in a large cohort of patients treated with widely used treatment regimens. Nevertheless, according to some smaller studies - both techniques have comparable prognostic significance. Nowadays, they are considered to be complementary tools for MRD monitoring^{6,12}.

Currently, new technologies for the detection of tumor-specific marker are emerging, such as droplet digital PCR or next generation sequencing (NGS) (ref.^{13,14}). However, the qualitative and quantitative detection of tumor-specific marker by ASO-PCR is still the golden standard technique⁷. Nevertheless, the technique of MM-specific marker detection using ASO-PCR has not been established in the Czech Republic so far. Therefore, the aim of this work was to introduce allele-specific qualitative and quantitative detection of MM-related marker by PCR on BM and peripheral blood samples of MM patients in our laboratory for further MRD assessment.

MATERIAL AND METHODS

Patients and samples

Frozen genomic DNA (gDNA) derived from mononuclear cells from BM (BMMC) of 10 newly diagnosed and relapsed MM patients diagnosed between 2006 and 2007 at the Faculty Hospital Brno was included in the study (Table 1). For 6 patients, gDNA samples of mononuclear cells from peripheral blood (PBMC) at the time of relapse were available, for 2 other patients, gDNA samples of BMMC at the time of relapse were available. gDNA was isolated using phenol-chloroform extraction, and stored at -20 °C. Also, gDNA from PBMC of 10 healthy donors was included in the study. This gDNA was isolated using QIAamp DNA Mini Kit (Qiagen). All samples were included only after patients signed the informed consent approved by Ethical committee of the hospital.

Amplification and sequencing of tumor-specific IgH gene rearrangement

To identify tumor-related IgH rearrangements, 500 ng of gDNA was PCR-amplified using sets of primers for IgH variable (V), diversity (D), and joining (J) gene segments with 2mM dNTP, 20 mM MgCl₂, 5x Buffer and GoTaq Flexi DNA Polymerase (Promega) (ref.^{15,16}). The reaction was carried out for initial denaturation at 94 °C for 1 min and then 33 (40) cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 10 min at 72 °C (ref.¹⁷). PCR products were then run on 2% agarose gels to find clonal products. Clonal PCR products were excised and purified using MinElute Gel Extraction Kit, QIAquick

PCR Purification Kit or QIAquick Gel Extraction Kit (all Qiagen) and further sequenced. Purified PCR fragments were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit on ABI3130 DNA Sequencer (Applied Biosystems). The relevant VH family or JH consensus primers were used as sequencing primers to obtain the sequence information (Table 2) (ref.^{15,16,18}).

ASO primers design and nested PCR amplification of tumor-specific IgH gene rearrangement

Patient-specific ASO primers of CDRIII region were designed using IMGT/V-QUEST (<http://www.imgt.org/>) and PrimerBlast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized by Eurofins MWG Operon (Ebersberg, Germany) (ref.¹⁹). Nested PCR amplification using ASO primers for each IgH sequence identified earlier were performed using 2mM dNTP, 20 mM MgCl₂, 5x Buffer and GoTaq Flexi DNA Polymerase (Promega). PCR was performed as initial denaturation at 94 °C for 1 min, and then 33 cycles of amplification at 94 °C for 30 s 58-62 °C (dependent on specific ASO primer T_m) for 30 s, and 72 °C for 30 s, with final extension of 10 min at 72 °C. PCR products were run on 2% agarose gels¹⁷.

Table 1. Patient's baseline characteristics.

Total number of patients	10
Sex: male/female	5/5
Average age at diagnosis (range) [years]	64 (52-81)
ISS stage: I-II-III (%)	60-30-10
Durie-Salmon stage: I-II-III (%)	40-0-60
Ig isotype: IgG-IgD-LC only (%)	70-10-20
Monoclonal Ig (g/l)	34.7 (0-79.8)
Plasma cells infiltration of bone marrow (%)	31.1 (10.0-81.6)

Table 2. Sequences of the consensual primers used for IgH rearrangement detection.

(Symbols: R = A/ G, Y = T/C, S = G/C, K = G/T).

Primer	Sequence (5'-3')
VH1FS	CAGGTGCAGCTGGTGCARYCTG
VH2FS	CAGRTCACCTTGAAGGAGTCTG
VH3FS	GAGGTGCAGCTGGTGSAGTCYG
VH4aFS	CAGSTGCAGCTGCAGGAGTCSG
VH4bFS	CAGGTGCAGCTACARCAGTGGG
VH5FS	GAGGTGCAGCTGKTGCAGTCTG
VH6FS	CAGGTACAGCTGCAGCAGTCAG
VHFR2-1	CTGGGTGCGACAGGCCCTGGACAA
VHFR2-2	TGGATCCGTCAGCCCCCAGGGAAGG
VHFR2-3	GGTCCGCCAGGCTCCAGGGAA
VHFR2-4	TGGATCCGCCAGCCCCCAGGGAAGG
VHFR2-5	GGGTGCGCCAGATGCCCGGGAAAGG
VHFR2-6	TGGATCAGGCAGTCCCCATCGAGAG
VHFR2-7	TTGGGTGCGACAGGCCCTGGACAA
JHD	ACCTGAGGAGACGGTGACCAGGGT

PCR products from ASO PCR were also sequenced as described previously, to ensure the detection of the same sequence.

Design of probes for RQ-PCR

For RQ-PCR analysis of tumor-related sequence H-chain V-region (VH) family-specific consensus reverse probes (called L-VH1 to L-VH6) derived from the germ-line sequence FR3 and designed for use in RQ-PCR in childhood ALL were used²⁰. Because of the high rate of somatic hypermutations occurring in MM, novel probes were required. The new specific IgH probes were designed according to recommendations of Ladetto et al.¹⁷ (Eurofins MWG Operon, Ebersberg, Germany) and labeled at the 5' end with 6-carboxy-fluorescein (FAM) and 6 carboxytetramethyl rhodamine (TAMRA) at the 3' end. RQ-PCR reaction was performed in 25 µL with 500 ng of patient's gDNA using 1x TaqMan Gene Expression MasterMix (Life Technologies), 10 pmol of each patient's specific ASO primer, 5 pmol specific IgH probe. Reactions were incubated in a 96-well optical plate at 50 °C for 2 min, 95 °C for 10 min, followed by 42 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate on 7500 Real-Time PCR System. Standards for RQ-PCR were obtained by cloning the tumor-specific IgH region with the TOPO TA cloning Kit (Invitrogen). A variable number of white-positive colonies (colonies with correct plasmid insertion) were grown overnight in Luria-Bertani broth containing 50 mg/mL ampicillin. Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen). Standard curves were prepared by ten-

fold serial dilutions of plasmid in gDNA obtained from healthy donors according to the European Study Group on MRD Detection in ALL (ESG-MRD-ALL criteria) (ref.²¹). Then, the quantitative analysis of tumor-specific sequence was related to the reference human RNase P gene (Applied Biosystems).

Monoclonality of the specific sequence was verified by sequence analysis of PCR product from single colonies cloned with specific VDJ gene rearrangement of IgH.

RESULTS

gDNA samples of ten patients obtained from BMMC were used for introduction of tumor-specific marker identification by PCR. Fifty percent of the patients (5/10) were suitable both for qualitative and quantitative tumor marker detection. In 30% (3/10) of patients, the sequence was not clear and they were assessed as oligoclonal. This technique was unsuccessful in 20% (2/10) of our patients (Table 3).

Tumor-specific IgH gene rearrangement amplification and sequence analysis

The tumor-specific marker was established for 80% (8/10) patients by PCR with consensual primers (derived from FR1 and FR2 conservative regions). IGHV3 allele was present in 30% (3/10) of patients, IGHV2 in 10% (1/10) and IGHV4 in 10% (1/10) of patients. Sequences were analyzed using bioinformatic tool IMGT/V-QUEST for CDR2/3 hypervariable region evaluation. Only pro-

Table 3. A summary of individual steps of PCR detection of tumor-specific marker in MM (nd – not done).

Patient	ASO PCR			qPCR		
	allele	ASO primers	PCR II	ASO probes	standard curves	qPCR
1	IGHV2	done	done	done	done	quantifiable
2	IGHV3	done	done	done	done	quantifiable
3	oligoclonal	done	done	nd	nd	nd
4	IGHV4	done	done	done	done	out of quantitative range
5	IGHV3	done	done	done	done	quantifiable
6	oligoclonal	done	done	nd	nd	nd
7	oligoclonal	done	done	nd	nd	nd
8	IGHV3	done	done	done	done	quantifiable
9	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	nd	nd	nd

Table 4. Results of RQ-PCR detection of tumor-specific marker in MM.

Patient	Status	ASO probe	RNase P	Malignant cells./ 10 ⁶ healthy cells	Conclusion
1	1. relapse	14.4	43 348	662,1	positive, quantifiable
	diagnosis	4 239	37 390	226 726,9	positive, quantifiable
2	1. relapse	1.7	29 462	114,4	positive, quantifiable
4	1. relapse	3.9	3 331,6	2 327	positive, out of quantitative range
5	diagnosis	21 963	59 797	734 598,4	positive, quantifiable
8	1. relapse	68	3 348,1	40 606,7	positive, quantifiable

ductive IgH rearranged sequences (in frame junctions, no stop codon) were used for further work.

Monoclonality of the specific sequence was verified by direct sequence analysis of cloned PCR product from plasmid in bacteria in 5 patients. In 3 remaining oligoclonal patients, we identified ≥ 3 different clones.

ASO primer design, testing and qualitative PCR for tumor-specific marker detection

ASO primers were designed for 5 patients with successfully obtained molecular marker and for 1 clone of 3 oligoclonal patients (Supplementary table S1). In 7 patients, ASO primers were designed according to patient's specific gDNA sequence and in 1 case, we used plasmid sequence with cloned VDJ region for primer design, because of higher quality of the sequence. Qualitative cross-reaction was performed to verify specificity of designed ASO primers to only one patient. Six out of 8 pairs of primers were specific for only one patient.

Qualitative PCR with designed ASO primers was performed on gDNA diagnostic samples from 5 patients with monoclonal sequence. The original clone was present in follow-up samples of 3 patients at the time of relapse. For the other 2 patients, samples were not available. Moreover, PCR was performed on follow-up samples at the time of relapse for 3 oligoclonal patients as well, where 1 of the original clones was successfully detected in 1 oligoclonal patient relapse sample.

ASO probe design and RQ-PCR for tumor-specific marker detection

Quantitative detection of MRD was performed on 5 monoclonal patients. In these patients, we were able to clone monoclonal sequence into plasmids in order to obtain standard curves (Supplementary table S2, Fig. S1). These standard curves fulfilled ESG-MRD-ALL criteria²¹. The detection was not performed for oligoclonal patients, as the plasmids carried different inserts of the VDJ rearrangements.

For RQ-PCR analysis of tumor-related marker, we started with specific consensus probes derived from the germline sequence of the FR3 region and designed for use in RQ-PCR in childhood ALL (ref.²⁰). These consensus probes were used in 2/5 patients (LVH2 and LVH3 probes) and were fully complementary with patient's sequences. Because of high rate of somatic hypermutations occurring in MM, we designed new probes according to recommendations of Ladetto et al. for 3/5 patients (Supplementary table S3) (ref.¹⁷).

Quantification was based on human RNase P reference gene. The RQ-PCR reaction was successful for all 5 patients. Four samples were assessed as positive (concordant with qualitative PCR results) and quantifiable (Table 4). In one patient, MRD was assessed as positive, out of quantitative range. The RQ-PCR sensitivity was up to 10^{-6} .

DISCUSSION

The aim of the work was to introduce MM-related marker identification for further MRD detection by PCR in our laboratory as a complementary tool for MRD assessment by FC. For the purpose of method introduction, we used retrospective patient samples from the time of diagnosis and relapse in order to confirm presence of clonogenic cells and their tumor-specific marker.

MRD monitoring is important for identification of patients at increased risk of relapse and plays a key role in treatment response assessment in clinical trials⁷. Unlike MRD detection by FC which can be applied in approximately 90% of patients with MM thus allowing routine examination, approaches based on PCR are more complex and can be applied in approximately 75% of patients with MM because of the extensive heterogeneity of the disease and presence of several MM clones at the time of diagnosis⁶. Although PCR is less applicable than FC, it is a powerful technique for treatment efficacy assessment and risk stratification in MM (ref.^{17,22-25}). In this study, with the PCR detection of clonogenic cells, we were able to obtain qualitative assessment in 80% of patients and quantitative data, important for serial monitoring, in 50% of patients (Table 3).

We preferentially used FR1 or FR2 derived primers, as we need to obtain sequence of variable regions CDR2/3. Therefore, FR1/2 derived primers allowed us to obtain sequence that was shorter and more suitable for our analysis compared to using L derived primers^{15,26}. Further, we did not use FR3 derived primers because the sequences obtained after such amplification are too short which increases the risk of false positive results. For comparison, Owen et al. used FR3 derived primers although this approach allowed detection of specific IgH rearrangement only in 56% of patients²⁷. However, all of the above mentioned approaches are possible, as described previously by van Dongen et al.¹⁶.

In our case, the successfully identified VDJ rearrangements were in accordance with average frequency of VH variants^{16,28}. Although it is possible to perform PCR reaction with several primer families, in approximately 20% cases we were not able to identify specific VDJ rearrangement sequence. This was most likely due to the presence of somatic hypermutations and subsequent loss of primer binding sites in patient-specific sequence given by extensive heterogeneity in MM clones^{4,7,15}. On average, there are 8% of mutated nucleotides of VDJ rearrangement sequence in MM patients^{29,30}; however only 2% in chronic lymphocytic leukemia (CLL) and 4% in follicular lymphoma⁷.

As the average homology of a patient's sequence with germline sequence is 92.2%, the annealing ability of primers derived from consensus regions of IgH is limited³⁰. In our case, the primer design was successful for all patients with monoclonal sequences (5/10), and for one of the clones of all patients with oligoclonal sequence (3/10). In 1/10 case, we used sequence obtained from the plasmid with cloned hypervariable region for the primer design, as was previously described by Voena et al.¹⁵.

We also verified specificity of designed primers to only one patient by qualitative cross-annealing reaction, as was shown in 6/8 designed pairs of primers. Specific ASO primers are used for tumor marker identification and its further detection during the patient follow-up. However, we cannot exclude annealing of specific ASO primers on different MM patient. The CDR2 and CDR3 regions are relatively short; therefore, there is a high probability of similar motifs repetition. Nevertheless, the crucial thing is that designed ASO primers are not able to amplify healthy DNA; primers have to be specific for MM (ref.³¹).

The method used for MRD quantification is RQ-PCR with ASO primers and probes. The fluorescence signal is provided by probe derived from the more conservative region of the VDJ rearrangement (FR3). First, the specific probe is chosen from previously designed probes for use in real-time PCR in childhood ALL (ref.²⁰). Since these probes can be used for several patients, there was a significant reduction in the price of this method compared to methods using patient-specific probes^{17,32}.

Concerning the probes, we confirmed results of Ladetto et al., who analyzed the effect of mismatch between probe sequence and sequence of the patient. Probe was unsuccessful every time in the case of at least three mismatches. In contrast, probe was always successful in the case of no or one mismatch. And in the case of two mismatches, the success of probe annealing was in the type of substitution: G/C (strong interaction); A/T (weak interaction) (ref.¹⁷).

In our study, the RQ-PCR was successful for all 5 monoclonal patients. Four samples were assessed as positive (concordant with qualitative PCR results) and quantifiable. In one patient, MRD was assessed as positive, but out of quantitative range. The rate of ASO RQ-PCR is considerably variable because of significant heterogeneity of MM cells but approximately ranging from 30% to over 80% (ref.^{22,23,32,33}). In the remaining 3 oligoclonal patients, we were not able to perform the quantification because of the oligoclonal nature of the disease. In these types of samples, it is not very feasible to prepare standard curves necessary for the MRD quantification.

The main advantage of MRD detection by RQ-PCR is its high sensitivity. The sensitivity is dependent on the specific ASO probe hybridisation and therefore also on the clone-specific IGHV sequence used for ASO primer and probe design¹⁷. Therefore, for this reason, we cannot reach the same sensitivity for all patients. In our work, we were able to reach RQ-PCR sensitivity of at least 10^{-4} and up to 10^{-6} . This result is in accordance with other studies since most studies dealing with RQ-PCR detection of MRD also reach sensitivity between 10^{-4} and 10^{-6} (ref.^{7,21,33}).

Despite all the risks and complications, PCR based MRD detection has wide application, such as quantification of tumor contamination in healthy hematopoietic cells for autologous transplantation or following the dynamics of clonogenic cells and activity of the tumor load^{34,35}. Its quantification is useful for treatment response and prognostic assessment as well as for early relapse detection^{17,22,23,36,37}.

CONCLUSION

Standard techniques used for remission evaluation are able to give only superficial information about the treatment efficiency because of their limited sensitivity. There is a need for more sensitive methods to gather more detailed insight and detection of small tumor cell residues. One of these methods is PCR detection of tumor-specific marker for MRD. This approach has some limitations because of significant heterogeneity of tumor plasma cells and presence of somatic hypermutations in MM. For this reason, it is only successful in some patients and also molecular biological approaches of MM-related marker detection are provided only in a limited number of laboratories. However, we successfully managed to establish this method at both qualitative and quantitative level in MM patients for the first time in the Czech Republic.

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Supplementary data

Table S1. Characteristics of used designed ASO primers for qualitative PCR detection of tumor-specific marker in MM.

Patient	Clonality	Allele	Primer	Sequence (5'-3')	Length (mer)	T _m (°C)
1	monoclonal	IGHV2	forward	CACTTATTGATTGGGATGGT	20	56
			reverse	TAGTCAAAGTGCAGTCGCT	19	56
2	monoclonal	IGHV3	forward	ATGAGTAGTGACGGGGGTA	19	58
			reverse	TGTTGAAACTCTCGCACAGT	20	58
3	oligoclonal	IGHV3	forward	TAGTGGAGGTGAAACCCAAT	20	58
			reverse	TGCTTCATCTCCAGTGCCA	19	58
4	monoclonal	IGHV4	forward	TTACACTGGGAGCACCAAC	19	58
			reverse	AGATCGTAATCCGATCTCGC	20	60
5	monoclonal	IGHV3	forward	AAATCACCACCCACGGAGAT	20	60
			reverse	TCCGACATCATAACGCACAGT	20	60
6	oligoclonal	IGHV3	forward	AGCTATATCACATGATGGAAGT	22	60
			reverse	TAGAACCCCCACTCCCGA	18	58
7	oligoclonal	IGHV1	forward	ACCCTAACGTTGGTGATACAA	21	60
			reverse	TAATCATAGTAATCTCTCGCAC	22	60
8	monoclonal	IGHV3	forward	TACTGGTGGTGGTAGCACAT	20	60
			reverse	CAATTATCATCCGCTTTCGC	20	58

Table S2. Standard curves parameters for RQ-PCR detection of tumor-specific marker in MM.

Patient	Probe	Slope	Correlation coefficient	Quantitative range	Sensitivity
1	ASO probe	-3.33	0.9988	1.00E-06	1.00E-05
	RNase P	-3.56	0.9995	1.00E-06	1.00E-05
2	ASO probe	-3.18	0.9959	1.00E-06	1.00E-06
	RNase P	-3.47	0.9991	1.00E-06	1.00E-06
4	ASO probe	-3.33	0.9959	1.00E-05	1.00E-05
	RNase P	-3.55	0.9985	1.00E-06	1.00E-06
5	ASO probe	-3.07	0.9985	1.00E-04	1.00E-04
	RNase P	-3.47	0.9990	1.00E-04	1.00E-04
8	ASO probe	-3.35	0.9974	1.00E-06	1.00E-05
	RNase P	-3.56	0.9995	1.00E-06	1.00E-05

Table S3. Sequences of designed probes for RQ-PCR detection of tumor-specific marker in MM.

Patient	Sequence (5'-3')	Length (mer)
2	5' FAM: CTCTGGAGATGGTGAATCTGCC-TAMRA	22
4	5' FAM: CCGTGTTTGC GGCGGTCACA-TAMRA	20
5	5' FAM: GCCGTGTCCTCGACCCTCA-TAMRA	19

Fig. S1. Standard curves for quantitative real-time PCR detection of tumor-specific marker in MM.

A) Patient 1, B) Patient 2, C) Patient 4, D) Patient 5, E) Patient 8.