Application of Self-Quenched JH Consensus Primers for Real-Time Quantitative PCR of *IGH* Gene to Minimal Residual Disease Evaluation in Multiple Myeloma

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Monitoring multiple myeloma patients for relapse requires sensitive methods to measure minimal residual disease and to establish a more precise prognosis. The present study aimed to standardize a real-time quantitative polymerase chain reaction (PCR) test for the *IgH* gene with a JH consensus self-quenched fluorescence reverse primer and a VDJH or DJH allele-specific sense primer (self-quenched PCR). This method was compared with allele-specific real-time quantitative PCR test for the *IgH* gene using a TaqMan probe and a JH consensus primer (TaqMan PCR). We studied nine multiple myeloma patients from the Spanish group treated with the MM2000 therapeutic protocol. Self-quenched PCR demonstrated sensitivity of $\geq 10^{-4}$ or 16 genomes in most cases, efficiency was 1.71 to 2.14, and intra-assay and interassay reproducibilities were 1.18 and 0.75%, respectively. Sensitivity, efficiency, and residual disease detection were similar with both PCR methods. TaqMan PCR failed in one case because of a mutation in the JH primer binding site, and self-quenched PCR worked well in this case. In conclusion, self-quenched PCR is a sensitive and reproducible method for quantifying residual disease in multiple myeloma patients; it yields similar results to TaqMan PCR and may be more effective than the latter when somatic mutations are present in the JH intronic primer binding site. (*J Mol Diagn* 2006, 8:364–370; DOI: 10.2353/jmoldx.2006.050101)

The configuration of immunoglobulin (Ig) genes shows a post-follicular origin in multiple myeloma plasma cells; these cells are somatically hypermutated and present no

intraclonal variation or class-switching.¹ After stem cell transplantation, 30 to 40% of multiple myeloma (MM) patients achieve complete remission (CR) with negative immunofixation, although most of them ultimately relapse.^{2,3} Monitoring of MM patient response to treatment has been classically based on the detection and quantification of patient M serum component. Accordingly, more sensitive methods are necessary to measure minimal residual disease (MRD) and to establish a more precise prognosis. Immunophenotypic flow cytometry studies of myelomatous plasma cells and the current molecular strategies are highly sensitive, allowing the detection of very low levels of MRD. However, both strategies have limitations.^{4–8}

Several real-time quantitative IgH polymerase chain reaction (PCR) assays have demonstrated optimal results for tumor burden quantification after treatment in patients with several hematological malignancies, including multiple myeloma.^{4,5,9–12} A major problem in multiple myeloma real-time PCR quantification is hypermutation in the VDJH rearrangement.

Measuring MRD by allele-specific oligonucleotide (ASO) real-time quantitative PCR for the *IgH* gene (IgH RQ-PCR) could be a good strategy to evaluate molecular response and its prognostic importance in MM patients who show complete remission on the immunofixation test.^{4,5} Usually, ASO primers are targeted to the VDJH junction, and the TaqMan probe and reverse primer are targeted to JH consensus and intronic regions. In this situation, it is possible that the consensus primers and probe coincide with a somatically mutated JH segment. If this happens, the primer and/or probe will be mismatched, and PCR efficiency will be dramatically reduced.⁵

The fluorescent techniques used in PCR-based detection are various: linear hydrolysis probes (TaqMan probes), hybridization probes, fluorescence resonance

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energy transfer-labeled oligonucleotides, molecular beacons, DNA-binding dyes (SYBRGreen), etc. A way to simplify real-time *IgH* gene PCR in postfollicular B malignancies is to avoid using a probe or to employ shorter probes. In this sense, several methods like SYBRGreen or short consensus probes have been proposed and used.^{13,14}

Nazarenko et al¹⁵ developed a novel fluorescent primer design that labeled primers with a single fluorophore on a base close to the 3' end. This technique does not require a quencher but is still suitable for real-time quantification and has demonstrated good results in quantifying *c-myc*, interleukin-4 cDNAs, and some reference genes. Other authors have used this approach to quantify the gene expression in neural precursors¹⁶ and to detect a gastroenteritis virus gene.¹⁷ But before now, this technique had never been used for *IgH* gene quantification.

The present study aimed at standardizing an ASO RQ-PCR test for the *IgH* gene with a JH consensus self-quenched fluorescence reverse primer and a VDJH or DJH allele-specific sense primer, called self-quencher PCR here, to quantify residual disease in MM patients after high-dose chemotherapy and then during follow-up. Results provided with this methodology were also compared with ASO IgH RQ-PCR using the TaqMan probe and JH consensus primer, which we will call TaqMan PCR.⁹

Materials and Methods

Patients and Samples

Bone marrow samples from nine MM patients were recruited for this study. All patients were treated according to the MM2000 therapeutic protocol proposed by the Grupo Español de Mieloma. This protocol consists of four cycles of VBCMP/VBAD (vincristine, BCNU, cyclophosphamide, melphalan, prednisone/vincristine, BCNU, adriamidine, and dexamethasone) alternate chemotherapy regimen followed by high-dose melphalan and autologous peripheral blood stem cell transplantation. All patients included in the study were in CR after transplantation. CR was defined by the electrophoretic disappearance of the M-component in both serum and urine (confirmed in two different samples obtained at an interval of 6 weeks), absence of soft-tissue plasmacytomas, a normal serum calcium concentration, stable skeletal disease, and less than 5% plasma cells in the bone marrow.³

The nine patients were randomly selected from a series of 57 patients achieving CR as previously defined, in whom the VDJH or DJH monoclonal rearrangement had been fully identified. The sequences of each rearrangement junction, ASO primers, and JH probes and primers are shown in Figure 1.

Methods

Patient samples were studied at diagnosis and at the time of the patient's best response to autologous peripheral

blood stem cell transplantation. Genomic DNA was isolated from 2 ml of bone marrow aspirates from each patient. High molecular weight DNA was isolated by standard proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation or automatic extraction with Magnapure (Roche Applied Science, Mannheim, Germany) based on magnetic bead technology.

Identification of the IgH clonal population was made according to the BIOMED II protocol.¹⁸ PCR product was directly sequenced first with the JH consensus primer and later with the VH primer, in an automated ABI 3100 Avant DNA sequencer using Big-Dye 3.1 terminator (Applied Biosystems, Foster City, CA). Germline VH, DH, and JH segments from complete VDJH rearrangements were identified by comparison with the V Base23 (<http://vbase.mrc-cpe.cam.ac.uk/>) and the International ImMunoGeneTics Information System database (<http://imgt.cines.fr>) using online DNAPLOT. Germline DH and JH segments from incomplete DJH rearrangements were identified using the BLAST search in the DH-JH germline locus sequence (accession no. EMB/X97051; <http://www.ncbi.nlm.nih.gov/blast/>) (Figure 1). The allele-specific primer for the CDR3 region of the IgH gene was designed according to previously described instructions.⁵

Self-Quenched PCR

The alternative method for assessing MM patient response to therapy was RQ-PCR with a consensus fluorogenic mono-labeled primer. This method used the same 5' allele-specific primer targeted for the VDJH or DJH CDR3 region as the TaqMan real-time PCR. The JH consensus primer designed by van Dongen et al¹⁸ was modified to design the self-quenched primer. The consensus JH primer consisted of a 3' primer labeled with a fluorescent dye and modified according to the general thermodynamic conditions previously described for the self-quenching primer in the Oligo 6 program¹⁹ (W. Rychlik, Molecular Biology Insights, Cascade, CO; <http://www.oligo.net>): the presence of either a C or G as the terminal 3' nucleotide of the primer and the fluorophore 6-FAM being attached to the last T base from the 3' end of the primer. The last step was the addition of a 5' tail, which is a complement sequence to the 3' end and thus forms a hairpin. The ΔG in the stem of the hairpin primers ranged from -1.6 to -5.8 kcal/mol.¹⁵ The final sequence of the self-quenched primer was GGTCACTTACCTGAG-GAGACGGTGACC (the primitive primer sequence is underlined, the 5'tail is represented in italics, and the position in which the fluorophore 6-FAM was attached is printed in bold type). The ΔG of the complementary sequence in the hairpin stem was -3.4 kcal/mol.

TaqMan PCR

Real-time quantitative PCR of the *IgH* gene was performed with TaqMan probes, using the previously published TaqMan and JH consensus primers⁹ and with the VDJH or DJH rearrangements as targets. This method was

Figure 1. Patient rearrangements and oligonucleotide sequences. Junction regions VDH, DHJ, or both are indicated with **vertical arrows**. N nucleotide insertions and somatic mutations are represented in lowercase letters. Allele-specific and JH intronic primer are **underlined with dotted lines**, probe sequences are **underlined with black lines**, and the self-quenched sequence is inside the **rectangle**. All cases have been sequenced using the JH consensus primer. Cases 10160, 9985, and 4526 (cases that did not work) have also been sequenced using the next JH intronic primer just downstream to study the mutations present in the probe and primer binding sites.

the control, and its protocols were adapted to the Light Cycler system (Roche Applied Science, Mannheim, Germany). A 5' allele-specific primer in the VDJH or DJH CDR3

region was designed for each individual patient, a specific TaqMan probe for the JH family and a 3'-specific JH intronic primer for the JH family were used for all patients.

Figure 2. Scheme and standard curves of two methodologies. **A:** Example of TaqMan PCR. Amplification curves of one patient's diagnostic sample, several dilutions to calculate standard curve, the sample after treatment (MRD), and the sample at the time of the relapse. NTC, nontemplate control. **B:** Example of self-quenched PCR. **C:** Melting curves in one self-quenched method case. There are two different melting temperatures: 84°C for the specific fragments (**B**) and 77°C for the nonspecific fluorescence (**A**).

Control Genes

Two different control genes were used for quantification result normalization: β -actin for the Taqman PCR method²⁰ and thromboxane A (TXA) for the self-quenched PCR, because the β -actin gene was not amenable to the modifications necessary to produce a self-quenched primer.¹⁸ The TXA forward primer sequence was modified according to the rules described above for self-quenched primer design. The primer sequences were TXAF, 5'-GGACTGCCCGACATTCTGCAAGTCC-3' and TXAR 5'-GGTGTGTCGGGAA GGGTT-3'. The ΔG of its complementary sequence was -1.9 kcal/mol.

PCR Conditions

Real-time PCR reactions were performed in a Light Cycler system using Fast Start Light Cycler TM DNA-Master containing Taq-polymerase, reaction buffer, and dNTPs. All reactions were performed in a 10- μ L volume with 500 ng of genomic DNA, 300 nmol/L each primer, 200 nmol/L TaqMan probe, and 4 mmol/L $MgCl_2$. To

optimize self-quenched PCR, it was necessary to adjust the primer concentration between 200 and 1000 nmol/L; the optimum concentration was 300 nmol/L in seven cases, 500 nmol/L in two cases, and 250 nmol/L in one case. For TXA PCR, the optimum primer concentration used was 500 nmol/L.

Cycling conditions were 10 minutes at 95°C for initial denaturation followed by 45 cycles of 0 seconds at 95°C and 30 seconds at 60°C. An annealing temperature of 65°C was used in two cases of ASO-PCR for the self-quenched PCR and also for the self-quenched PCR for the TXA gene. When self-quenched primer was used, the PCR products were subjected to a melting cycle with a cooling ramp rate of 0.2°C/seconds from 95 to 70°C, with continuous monitoring of the fluorescence ratio to establish the T_m of the amplified fragment (Figure 2).

The diagnostic DNA from each patient was serially diluted down to 10^{-5} in 10-fold increments into polyclonal DNA from healthy individuals (Figure 2). Standard curves were calculated using these dilutions. Successful RQ-PCR was assessed according to a standard curve with a

Table 1. Results of MRD Calculated by Two Methods

No.	Efficiency	Slope	Sensitivity	<i>r</i>	MRD	Copies/cells
1822 TaqMan	2.14	−3.03	5×10^{-5}	−0.98	2.8×10^{-4}	4
1822 Self-q	1.92	−3.54	5×10^{-5}	−1.00	3.8×10^{-4}	4
10084 TaqMan	1.74	−4.14	5×10^{-4}	−0.99	1.2×10^{-2}	4
10084 Self-q	2.12	−3.07	10^{-4}	−0.99	5.6×10^{-2}	8
10305 TaqMan	1.99	−3.35	5×10^{-5}	−1.00	6.6×10^{-2}	4
10305 Self-q	1.71	−4.29	10^{-4}	−0.99	2.7×10^{-2}	8
10160 TaqMan	1.95	−3.45	10^{-3}	−0.99	N.R.	—
10160 Self-q	1.59	−4.97	10^{-2}	−0.98	N.R.	—
13349 TaqMan	2.88	−2.18	10^{-4}	−0.98	1.3×10^{-2}	8
13349 Self-q	1.89	−3.63	5×10^{-4}	−0.99	1.8×10^{-2}	16
7509 TaqMan	2.26	−2.82	10^{-4}	−0.99	2.2×10^{-4}	8
7509 Self-q	2.01	−3.3	5×10^{-4}	−0.98	1.9×10^{-4}	16
2262 TaqMan	2.13	−3.04	5×10^{-4}	−0.99	7.5×10^{-1}	16
2262 Self-q	2.05	−3.21	5×10^{-4}	−0.99	7.1×10^{-1}	16
9985 TaqMan				−0.96	N.A.	—
9985 Self-q	2.14	−3.03	10^{-4}	−1.00	N.A.	8
4526 TaqMan				−0.88	N.R.	—
4526 Self-q					N.R.	—

Characteristics of standard curves, sensitivity and efficiency in every case. *r*, correlation coefficient; N.A., results not available because there is no MRD sample; N.R., no results because of technique failure.

correlation coefficient (*r*) of at least 0.98, and the specific fluorescence quantification was calculated with the aid of the Light Cycler software v3.01. PCR efficiency was calculated according to the formula: $\text{PCR } E = 10^{-1/\text{slope}}$. Relative quantification of MRD was performed according to the following formula from Pfaffl et al.²¹

$$\text{IgHMRD} = \frac{(E_{\text{IgH}})^{\Delta \text{CT}_{\text{IgH}}(\text{Diagnosis-MRD})}}{(E_{\text{control}})^{\Delta \text{CT}_{\text{control}}(\text{Diagnosis-MRD})}}$$

Intra- and interassay reproducibility was assessed by repeating the same experiment five times. Maximal sensitivity was defined as the last dilution of diagnostic DNA in which at least one of the duplicate dilution samples gave a positive fluorescent signal with a maximum C_T value of 40 cycles. This C_T value had to be at least three cycles lower than the C_T values found in any unspecific amplification with polyclonal DNA.

When the ASODJH or DJH PCR for both techniques failed (sensitivity below 5×10^{-4} or a slope ≥ 4), a larger fragment using the JH intronic primer just downstream was amplified and sequenced to find possible mutations in the probe or the JH binding site. The MeltCalt program was used to estimate the changes that somatic mutations produced in the ΔT_m for each probe and primer in VDJH rearrangements.²²

Results

In six of the nine patients, it was possible to calculate the MRD with both methods using standard curves. The parameters of the standard curves and results for each case are shown in Table 1.

Self-Quenched PCR

Self-quenched PCR worked in seven cases. The sensitivities of the standard curves varied from 5×10^{-4} , or 16 copies of the target gene, to 5×10^{-5} , or 4 copies of the

target gene. This is based on the assumption that a cell contains 6.25 pg of DNA and the fact that 0.5 μg of DNA was used per test. PCR efficiencies varied from 1.71 to 2.14 (Table 1). Intra-assay reproducibility for the cycle threshold (C_t) of 8800 copies had a mean and SD of 31.72 ± 0.13 , reflecting a coefficient of variation (CV) of 0.43%, whereas the interassay reproducibility for the C_t of 8800 copies was 31.86 ± 0.41 and CV 1.3%. A melting curve was performed at the end of PCR cycling to check the specificity of the amplifications. The T_m for specific products varied from 76 to 83°C. Unspecific amplifications due to primer dimmer formation were identified by the presence of different melting peak T_m s (Figure 2).

The control gene for this approach was *TXA* gene, modified as a self-quenched primer as previously described in Methods. The efficiency of the self-quenched PCR for the *TXA* gene was 1.87, the slope was −3.67, and the correlation coefficient was equal to 1. The mean and SD for the intra-assay reproducibility of the 16,600 copy C_t was 26.67 ± 0.38 , with a CV of 1.45%, and the interassay reproducibility for the 16,600 copy C_t was 28.26 ± 0.56 and CV was 2%.

TaqMan PCR

TaqMan PCR worked in six cases. The sensitivities of the standard curves ranged from 5×10^{-4} , or 16 copies of target gene, to 5×10^{-5} , or 4 copies of target gene. PCR efficiencies ranged from 1.74 to 2.88. Intra-assay reproducibility for the 8800 copy C_t had a mean and SD of 29.10 ± 0.31 , reflecting a CV of 1.05%; and interassay reproducibility for the 8800 copy C_t was 29.79 ± 0.8 and CV was 2.8%.

This approach used β -actin as the control gene. Each primer (0.3 $\mu\text{mol/L}$) and a 60°C annealing temperature were used for this PCR. The efficiency was 1.96, the slope was −3.41, and the correlation coefficient was equal to 1. The intra-assay reproducibility for the 16,600

copy Ct had a mean and SD of 24.82 ± 0.08 , reflecting a CV of 0.35%, and the interassay reproducibility for the 16,600 copy Ct was 23.52 ± 0.27 with a CV 1.16%.

The Comparison of Self-Quenched and TaqMan PCR

Sensitivity ranged between 5×10^{-4} and 5×10^{-5} with both methods. The TaqMan probes provided better sensitivity in three patients whereas the self-quenched primer was more sensitive in a different patient (Table 1). PCR efficiency ranged from 1.71 to 2.14 using the self-quenched primer and between 1.74 and 2.88 using the TaqMan probe (Table 1).

In one case, the self-quenched primer technique was effective, but the TaqMan probe technique failed, while in another two cases, the efficiency and/or sensitivity were inadequate with both methods.

Minimal residual disease could be measured with both methods in six of nine patients. Results were similar in all six patients, and these all had the same MRD logarithm with the two proposed methods (Table 1).

Causes for Unsuccessful ASO RQ-PCRs

As previously mentioned, the efficiency and sensitivity of PCR were inadequate in three cases. This was due to the presence of somatic mutations in the target for the probe, the JH intronic primer, or the JH self-quenched primer (Figure 1).

In case 9985, the self-quenched PCR provided good results, but the TaqMan PCR approach did not work. The VDJH sequence revealed that zone binding with the JH intronic primer had two mismatches that produced a ΔT_m of 9.8°C. In this case, the self-quenched approach yielded a correct amplification despite the presence of a mismatch in its primer binding zone. This mismatch produced a ΔT_m of 3.6°C.

In another case, 4526, the TaqMan approach had a total of six mutations, three in the probe, and three in the JH intronic primer binding site with a ΔT_m of 14.9°C. The self-quenched primer binding site had one mutation with a ΔT_m of 3.4°C, but it was located just at the 3'-terminal nucleotide of the primer.

Finally, case 10160 had a somatic mutation in the JH intronic primer binding site resulting in a ΔT_m of 8.5°C, which explained the very low sensitivity for the TaqMan approach. In this case, the self-quenched method did not work either. The JH self-quenched primer binding site showed only one mutation with a ΔT_m of 5°C and did not show primer dimer formation. This ΔT_m , although not high, was the only explanation we found for this inadequate result.

Discussion

The present study has evaluated a new methodology for molecular evaluation of residual disease in MM patients that shows some advantages over other methodologies.

The most important being a need for only two primers to get a specific PCR quantification of the monoclonal hallmarks in MM patients. This makes it possible to avoid problems derived from the use of the probes that are required in other methodologies, without a significant loss in the reproducibility, sensitivity, or specificity of the procedure.

Molecular detection techniques, like PCR amplification of clonal VDJH rearrangements, are very sensitive but methodologically difficult.^{5,6,8,23,24} Until now, ASO IgH TaqMan PCR, or TaqMan PCR, has been the most widely accepted method for evaluating MRD in MM patients.^{5,6,24} TaqMan PCR has been widely accepted for many diseases^{9,12,25} and is now being introduced in MM.^{4,5,8} This technique has demonstrated its usefulness in lymphoid malignancies that present no recurrent translocations because they are of pre-follicular origin. However, it is not always possible to apply this approach to mature postgerminal B lymphoid malignancies, like MM, because of the frequent presence of somatic mutations in the *IgH* gene.^{5,9,12,26} Several authors have suggested that a mutation in the JH primer or probe binding site with a $\Delta T_m > 6^\circ\text{C}$ or the presence of more than three mutations are always associated with PCR failure.^{5,6}

Here, we have designed a single JH consensus fluorescent primer and optimized an ASO RQ-PCR for real-time quantification of VDJH clonal rearrangements in multiple myeloma in a technique that is called self-quenched PCR. This strategy allows the use of PCR with only two oligonucleotides (the classical forward and reverse), avoiding problems derived from the use of a third oligonucleotide (the probe). In its place, the self-quenched JH consensus primer can act as the specific molecular dye for the reaction. This strategy will resolve some problems derived from the existence of somatic mutations. An example of this difficulty with somatic mutations would be the results in patient 9985. Using a self-quenched primer avoided the use of the JH intronic 3' primer, the binding site of which was strongly mutated. In contrast, the sensitivity, reproducibility, quantification curve efficiency, and molecular MRD levels with both methods were very similar (almost identical) in six patients (Table 1). In addition, the two control genes, β -actin in the TaqMan PCR and TXA in the self-quenched PCR, provided a similar level of VDJH-specific gene segments for each patient. Thus, both methods are equivalent in measuring the DNA quality.

Another advantage of the self-quenched method is cost. This approach only needs the ASO-specific primer and one fluorescent JH consensus primer that can be used for all patients. In contrast, TaqMan PCR requires three different consensus probes and six different specific JH intronic primers. By our estimate, self-quenched PCR costs about 20 to 25% less than TaqMan PCR.

In summary, ASO RQ-PCR of VDJH clonal segments using a self-quenched primer, or self-quenched PCR, is a reproducible and sensitive method for evaluating MRD in multiple myeloma patients achieving CR after high-dose therapy and stem cell transplantation. This methodology provides results comparable with TaqMan strategies,

and it could be even better in the cases with frequent somatic mutations in the JH intronic primer binding site.

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