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The use of fluorescent molecular beacons in real time PCR of IgH gene rearrangements for quantitative evaluation of multiple myeloma

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Summary

Background and objective: Fluorescent molecular beacons have been employed as hybridization probes in real time quantitative PCR to quantify residual disease in multiple myeloma (MM). *Design and methods*: After clinical diagnosis of MM, the CDR1, CDR2 and CDR3 regions of the IgH gene were analysed and sequenced to identify its clonal nature. Unique sequences of the clonal IgH rearrangement were used to design specific molecular beacon probes for each MM patient. A molecular beacon probe for the β-globin gene was used as a reference control to calculate relative amounts of the clonal B-cell population. *Results*: Optimization of probe design resulted in the use of a competitive sequence at the IgH area target between the loop and part of the stem of the molecular beacon. Cycling conditions and fluorescence temperature acquisition were optimized for a Light Cycler™. To validate this method for the follow-up of treated MM patients, we investigated accuracy, as well as interassay and intrassay reproducibility. *Conclusions*: Our results indicated that real time PCR with specific molecular beacons provides a feasible, accurate and reproducible method for the determination of minimal residual disease in MM.

Keywords Multiple myeloma, IgH gene, real time PCR, molecular beacon

Introduction

Since most multiple myeloma (MM) patients show persistence of residual disease after intensive treatment, the development of new techniques to distinguish different prognostic groups is an area of great interest. 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 (Billadeau *et al.*, 1997). The monitoring of MM treatment has been approached using a variety of different PCR procedures that do not allow for comparison of prognostic information (Billadeau *et al.*, 1997; Rawstron *et al.*, 1997; Szczepek *et al.*, 1998; Corradini *et al.*, 1999; Cavo *et al.*, 2000; Cremer *et al.*, 2000; Lopez-Perez *et al.*, 2000; Martinelli *et al.*, 2000a,b).

Conventional PCR of the IgH gene in the majority of MM patients in complete remission after autologous stem cell transplantation identifies a single clonal population (Davies *et al.*, 2001). This probably reflects the high level of disease in these patients. The recent development of real time PCR, with its accuracy, specificity, reproducibility and interlaboratory comparability, offers new insights into the field of minimal residual disease determination.

Molecular beacons are hairpin shaped oligonucleotides with an internally quenched fluorophore, the fluorescence being restored on binding to a target sequence in the PCR product (Tyagi & Kramer, 1996; Heyduk & Heyduk, 2002). 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 the annealing of self-complementary arms at the 5′-and 3′-ends of the probe. These arms are typically G + C rich to increase bond strength in the short sequence stretch of the stem.

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The aim of the present study was to standardize a real time quantitative PCR test for the IgH gene using alelle-specific molecular beacons as fluorescence probes to quantify residual disease in MM patients during follow-up after treatment with high dose of chemotherapy.

Patients and methods

Patients

The two patients included in this study were diagnosed with MM using standard clinical criteria (Kyle, 1992) and were subsequently included in a treatment protocol involving double transplantation (Lahuerta-Palacios *et al.*, 1997).

PCR analysis and sequencing

Genomic DNA was isolated from bone marrow at diagnosis using conventional protocols. A previously described PCR method for the CDR1, CDR2 and CDR3 region of the IgH gene (Aubin *et al.*, 1995) was used to identify clonal populations of plasma cells. To obtain the clonal sequence of the IgH gene, the PCR fragments obtained were cloned into pGEM-T (Promega, Madison WI, USA), and sequenced automatically.

Primers and probes

The IgH primers employed were Fr1c 5′-AG GTGCAGCTGG/CA/TGG/CAGT CA/G/TGG-3′, Ca2 5′-AA CTGCTGAGGAGACGGTGACC-3′. The molecular beacon sequences were 5′-CCCCCCTTGGCCCCAGACGTCCGGG GGG-3′ and 5′-CCGGGGGCAGCTGGTACTACCACAATA GTCCCCCGG-3′ for patients 1 and 2, respectively. The β-globin primers were globin-F 5′-ACACAACTGTGTTCAC TAGC-3′ and globin-R 5′-CAACTTCATCCACGTTCACC-3′ and the molecular beacon was 5′-CGCGCGGAGAAG TCTGCCGTTACTGCCCTGCGCGCG-3′ (Gelmini *et al.*, 1997). All molecular beacons were labelled at the 5′ end with 6-carboxy-flourescein (FAM) and at 3′ end with Dabcyl (Cruachem, Glasgow, UK).

Real time quantitative PCR

Duplicate PCR amplifications were carried out in a Light Cycler^{TM} (Roche Biochemicals, Mannheim, Germany) using Fast Start Light Cycler^{TM} DNAMaster containing Taq-polymerase, reaction buffer and dNTPs (Roche). 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).

For real time PCR of IgH: 1.5 μM of each primer and 0.5 μM of the corresponding molecular beacon was used. The amount of fluorescence signal was affected by $MgCl_2$. The optimal $MgCl_2$ concentration for the assay was 4 mM for patient 1 and 4.5 mM for patient 2. Optimal fluorescence acquisition was determined experimentally for each probe, which in turn required modification of the cycling programme. Cycling conditions for patient 1 were: 45 cycles of denaturation (94 °C/0 s), fluorescence acquisition (55 °C/0 s), annealing (62 °C/10 s) and elongation (72 °C/12 s). Conditions for patient 2 were: 45 cycles of denaturation (94 °C/0 s), annealing and fluorescence acquisition (62 °C/10 s) and elongation (72 °C/12 s).

For real time PCR of β-globin, 1 μM of each primer (globin-F and globin-R) and 0.5 μM of the corresponding molecular beacon were used at 4 mM $MgCl_2$. Cycling conditions were 45 cycles of denaturation (94 °C/0 s), annealing and fluorescence acquisition (60 °C/10 s) and elongation (72 °C/12 s).

Standard curves for quantification were prepared by making serial 10-fold dilutions of the plasmid containing the rearranged clonal IgHa from each patient and the purified β-globin fragment PCR product.

Results and discussion

The PCR analysis of CDR1, CDR2 and CDR3 regions of the IgH gene after the clinical diagnosis of MM in our patients revealed clonal IgH rearrangements, as indicated by the presence of single bands on electrophoresis (Aubin *et al.*, 1995). These single PCR fragments were cloned and sequenced, allowing the design specific molecular beacon probes for each MM patient. The sequences obtained were unique for each patient. The use of consensus sequences to design fluorescent probes is usually unsuccessful because MM is a differentiated B-cell malignancy, in which the VH–DH region of IgH gene may have many rearrangements. A consensus sequence for a group of patients is hardly ever found. In our study, the IgH probes were designed in the CDR3 region, where the patients showed highly specific sequence rearrangements. The probes were designed with an initial approach of optimizing the area for amplification using Primer Express software (PE Biosystems, Foster City, CA, USA). A second step in designing the probes was calculation of the melting temperatures of the different parts of the molecular beacon, according to Figure 1a. For optimal probes, the overall melting temperature should be 17–19 °C higher than the melting temperature of the loop. In designing patient-specific probes, we observed improved specificity when the G + C rich stem contained a sequence included

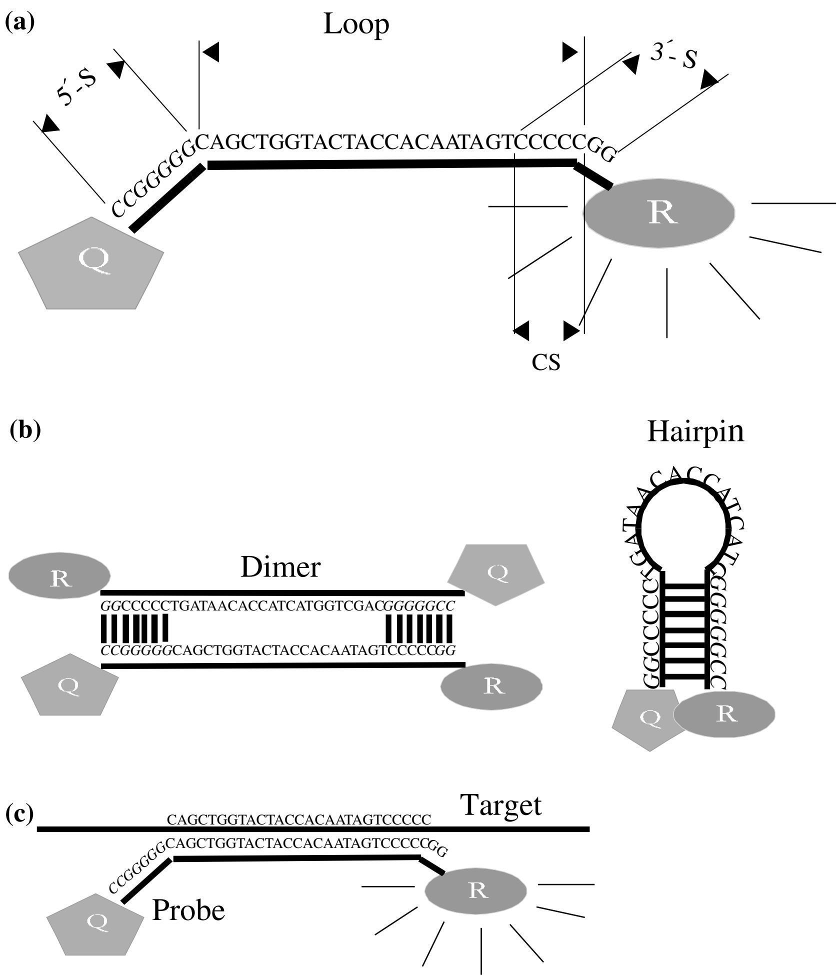

Figure 1. Operation of the molecular beacon. (a) Molecular beacon structure: the loop is complementary to the target sequence; the stem facilitate the formation of a self-complementary structure; and the competitive sequence belongs to both loop and stem. (b) The molecules do not light up, because the molecular beacon is self-complementary in the absence of the target (either by forming a hairpin or a dimer) and the fluorophore is quenched. (c) When the probe sequence hybridises to its target, forms a stable hybrid that takes apart the quencher from the fluorophore, which lights up reporting the presence of the target. Abbreviations: Q, quencher; R, reporter; S, stem; CS, competitive sequence.

in the target sequence in the DNA fragment corresponding to the clonal rearrangement (Figure 1a). Once designed, the molecular beacon sequence was analysed using Gene Bank software to test its unique specificity within the human genome.

Important experimental conditions influencing probe behaviour were fluorescence acquisition temperature and $MgCl_2$ concentration. When the experimental melting temperature of the beacon loop was lower than the acquisition temperature in the PCR programme, a fluorescence signal was not detected during cycling and it was necessary to decrease the acquisition temperature to values close to the melting temperature of the loop.

In our PCR assays of IgH using molecular beacons, the maximum sensitivity by serial dilution was 1×10^2 copies/μl. Intra-assay reproducibility of the crossing threshold (CT) was performed using seven replicate analyses. The coefficient of variation (CV) at 6×10^7 copies/μl was 2.7% and a similar value (2.9%) was obtained at 6×10^6 copies/μl. The CV for β-globin was 3.3% at 1×10^9 copies/μl (Figure 2). Inter-assay CT reproducibility over four different days was 4.63% for β-globin and 6.15% for patient 1 IgH (Figure 3).

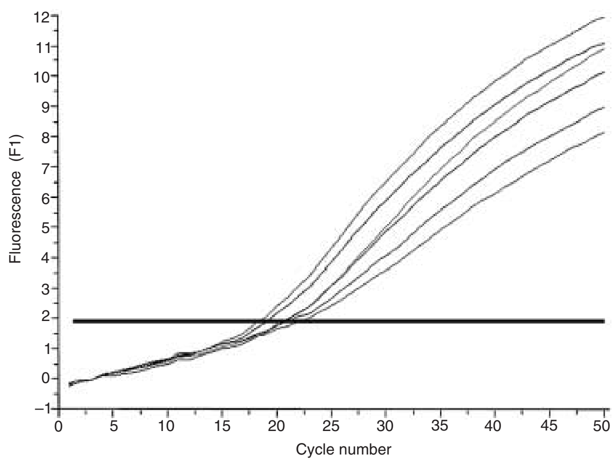

Figure 2. Intra-assay reproducibility of patient 1 IgH PCR. Crossing point of six replicates on the same experiment. CV of crossing point at an IgH of 6×10^7 copies/μl was 17.5 ± 0.45.

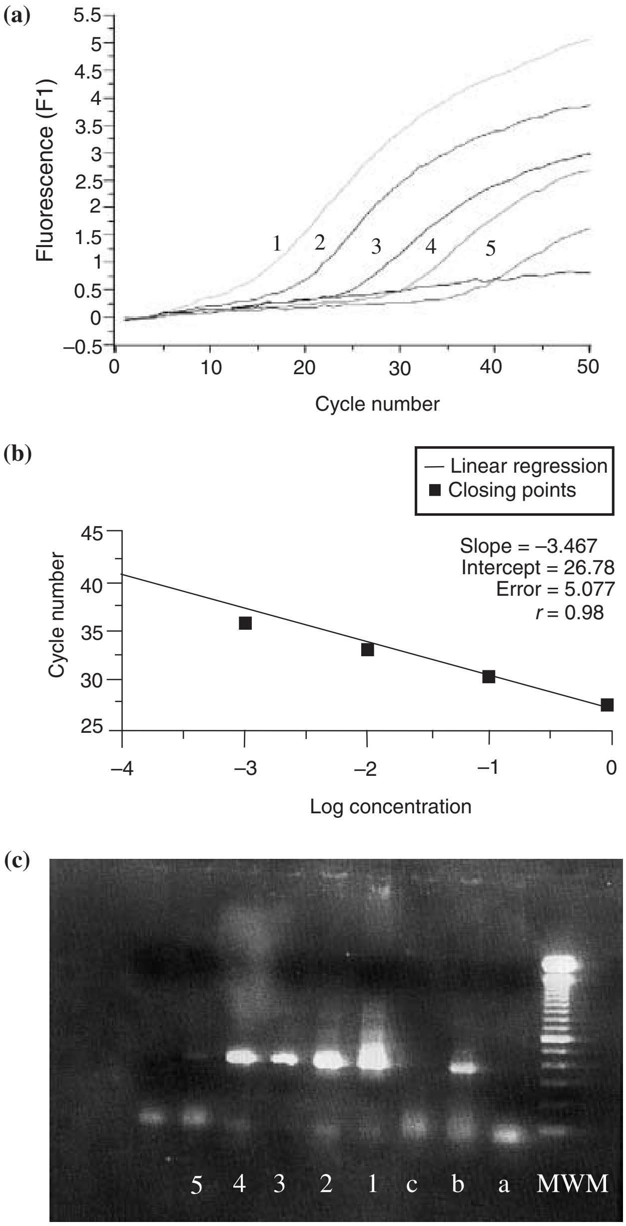

Figure 3. (a) Patient 1 IgH standard curve: plasmid dilution from 6×10^7 copies/μl to 6×10^2 copies/μl. (b) Linear regression of the standard curve: Intercept (Y) is the log of the amount of DNA at threshold divided by log of the efficiency; the slope (S) is −1/log efficiency; and R, linear regression coefficient. (c) 2% gel electrophoresis of the IgH standard curve from (a), where 1–5 are the corresponding 1–5 dilutions shown in (a); *a* is a no template control; *b* is another MM patient with different clonal IgH rearrangement; *c* is a IgH polyclonal control, *MWM* is a molecular ladder of 100 bp increase in size.

Both MM patients studied were in partial remission after two autologous stem cell transplants (ASCT). In both patients, the tumour load decreased one log after the first ASCT, but did not show any further decrease after the second. Although these patients did not achieve complete remission, their disease status correlated well with the results of molecular beacon real time PCR during the course of treatment.

The achievement of complete response is the main objective in myeloma treatment, as in the treatment of other hematological malignancies. With more effective treatments, the rate of complete response in myeloma patients has increased, although the cure rate remains low and most patients relapse. The study of minimal residual disease in MM is restricted primarily to those patients who achieve remission after treatment. In this group, patients with a better prognosis may be identified by a lower level of minimal residual disease. This may improve prognostic classification, enabling individualized treatment (Corradini *et al.*, 1999). Neither of the two patients in this study achieved complete remission. However, the study has enabled us to optimize this molecular technique for future application.

Our results indicate that real time PCR of the IgH gene using allele-specific molecular beacons provides an accurate and reproducible method for the determination of minimal residual disease in MM, as do other methods of IgH real time PCR described for other clonal B-cell malignancies (Pongers-Willemse *et al.*, 1998; Ladetto *et al.*, 2000; Verhagen *et al.*, 2000). Our results further indicate that when consensus probes and oligospecific primers cannot be used to quantify minimal residual disease in MM, allele-specific molecular beacons can be used effectively for this purpose.

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