

Assessment of IgH PCR strategies in multiple myeloma

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Abstract

Aims—To compare the ability of four commonly used PCR techniques to demonstrate clonal IgH rearrangements in multiple myeloma.

Methods—Bone marrow samples (containing a minimum of 10% plasma cells) were obtained from 127 patients with confirmed multiple myeloma. Framework 3 (Fr3) PCR was performed in all cases and the Framework 1 (Fr1f) PCR, which utilises six VH family specific primers, in 98 cases. In addition, 44 cases were assessed by Fr3, Fr1f, Framework 2 (Fr2) and Framework 1 consensus (Fr1 con) PCR techniques. JH primer selection was also assessed such that each PCR strategy was performed twice in each of the 44 cases, using the JH consensus primer (JH con) alone and then repeated with an equimolar mixture of JH con, JH3 and JH6 (JH mix).

Results—Clonal rearrangements were demonstrated in 71 (56%) of 127 cases with the Fr3 PCR and in 52 (53%) of 98 with the Fr1f PCR. However, by using both techniques it was possible to demonstrate clonal IgH rearrangements in 92 (75%) of 122 cases. Forty four cases were assessed by all four PCR techniques; in these cases the Fr3 and Fr1f PCRs demonstrated clonal rearrangements in 26 (59%) cases with a combined yield of 34 (77%). The Fr2 and Fr1 con PCR techniques had inferior pick up rates, demonstrating clonal rearrangements in 21 (48%) of 44 cases and a combined yield of 28 (63%). The Fr2 PCR did, however, demonstrate a clonal rearrangement in one case negative by both Fr3 and Fr1f. Two additional rearrangements were demonstrated by using JH mix; one became positive by Fr3, Fr1f and Fr2 and the other positive by Fr1f, Fr1 con and Fr2.

Conclusion—By utilising both the Fr3 and Fr1f PCR techniques it is possible to demonstrate definitive clonal rearrangements in the majority of patients with multiple myeloma. The Fr1 con and Fr2 PCR techniques have inferior pick up rates but may detect some additional rearrangements.

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myeloma has resulted in a necessity for molecular monitoring during the course of the disease. The absence of a disease specific chromosomal marker means that methods which use PCR to detect clonal rearrangements at the immunoglobulin heavy chain (IgH) locus are the most applicable for the assessment of residual disease. A number of methods have been described to date; the majority use a consensus primer from the JH region along with another primer from a variety of locations along the VH region (fig 1). The most commonly used technique was described by Brisco *et al*¹; it utilises another consensus primer from the third framework region. Alternative techniques utilise either six VH family specific primers from the first framework region² or a single consensus primer from the second framework region.^{3,4} A Framework 1 consensus (Fr1 con) technique has been described recently; it utilises a single consensus primer which binds to a highly conserved site located approximately 30 bases upstream from the Fr1f binding site.⁵

The ability to demonstrate clonal IgH rearrangements varies between lymphoproliferative disorders and is also dependent on the PCR strategy used. Yields of up to 90% have been reported in acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL)^{4,6} but significantly lower yields have been reported in multiple myeloma and follicular lymphoma.^{1,7} The detection rate in multiple myeloma and follicular lymphoma may be improved by utilising the Fr2 and Fr1 con PCR techniques.^{3,5} It has also been suggested recently that the failure to demonstrate clonal rearrangements may be due to the fact that some JH primers have insufficient homology at their 3' ends with JH3 and JH6 segments.⁵ It is clearly desirable to use the techniques which maximise the yield of clonal rearrangements; this is largely unknown in multiple myeloma as the majority of studies to date have looked predominantly at ALL and CLL or have looked at insufficient numbers of patients with multiple myeloma. In this study we have therefore assessed all four IgH PCR strategies in a large series of patients and have also assessed the effect of JH primer selection on the ability to demonstrate clonal rearrangements.

Methods

All the patients included in this study were diagnosed as having multiple myeloma by standard criteria and monoclonality was confirmed by the presence of circulating paraproteins.

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The advent of autologous and allogeneic transplantation as treatment options in multiple

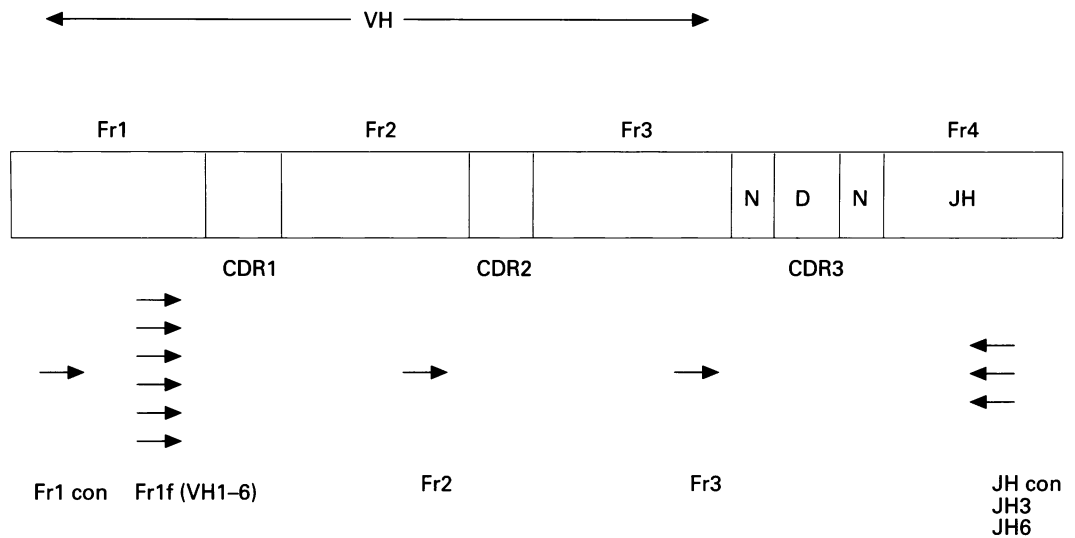


Figure 1 Schematic representation of a rearranged IgH gene. The primer binding sites for each of the four PCR techniques used in the present study are indicated.

tein or monoclonal cytoplasmic immunoglobulin, or both, within the bone marrow plasma cells. High molecular weight DNA was obtained from diagnostic bone marrow samples (containing at least 10% plasma cells) by proteinase K digestion, phenol/chloroform extraction and cold ethanol precipitation. The Fr3 PCR was assessed in 127 cases and the Fr1f PCR in 98. In addition, 44 cases were assessed by Fr3, Fr1f, Fr1 con and Fr2 PCR techniques. JH primer selection was also assessed such that each PCR technique was performed twice for each of the 44 cases using either the JH consensus primer alone or an equimolar mixture of JH consensus, JH3 and JH6 primers.

The primers used are outlined in table 1; the JH primers were 5' end labelled via an amino-hexyl link with a green fluorochrome. PCR reactions were performed in 25 µl volumes with 1 µg of DNA, 10 picomoles of sense and antisense primer in buffer containing 10 mM Tris (pH 9.1), 500 mM KCl, 0.25 mM each dNTP, and 1.5 mM MgCl₂. When the mixture of JH primers was used, 5 picomoles of each was included per PCR reaction. The reaction mixtures were held at 95°C prior to the addition of 1 unit of Taq polymerase (Super-Taq, HT Biotechnology Ltd., Cambridge, UK). Conditions for Fr3 PCR amplification were: 95°C for one minute, 60°C for one minute and 30 seconds for a total of 35 cycles followed by 10 minutes at 72°C. Conditions for Fr1f, Fr1 con and Fr2 PCR amplification

were: 95°C for one minute followed by 68°C for one minute (reduced in consecutive cycles by 1°C to 62°C) and 72°C for one minute for a total of 35 cycles, followed by 72°C for 10 minutes. The products of PCR amplification were then analysed as follows: 1 µl of each PCR product was added to 4 µl of loading mixture consisting of 3 µl formamide, 0.5 µl red fluorochrome labelled size standard (GS 2500 ROX, Applied Biosystems) and 0.5 µl dextran blue. Each mixture was then denatured at 95°C for three minutes and held at 4°C prior to loading onto 6% polyacrylamide gels, which were then analysed using an Applied Biosystems automated DNA sequencer (model 373A). The associated collection and analysis software is able to convert the gel image for each sample into an "electrophoretogram" which consists of number of peaks whose height correspond to the intensity of fluorescence on the gel. A polyclonal pattern appears as a number of peaks separated by 3 base pairs (bp) and arranged in a normal distribution, whereas clonal rearrangements appear as distinct peaks. The software is able to size rearrangements consistently to within a single bp and is also able to size each peak within a fingerprint, which greatly facilitates the identification of clonal rearrangements of known size when they are present within a polyclonal background. Sensitivity was assessed to be at least 10⁻³ for each of the four techniques and control PCRs were not performed as polyclonal fingerprints were demonstrable in all "negative" samples.

Table 1 Primer sequences used in each PCR strategy

Primer	Sequence
Fr3	5' CCGAGGACACGGC(C/T)(C/G)TGTATTA CTG 3'
Fr1f: VH1	5' CCTCAGTGAAGGGTCTCCTGCAAGG 3'
VH2	5' GAGTCTGGTCTGCGCTGGTGAAA 3'
VH3	5' GGTCCTGAGACTCTCCTGTGCA 3'
VH4	5' TTCGGA(G/C)ACCCTGTCCCTACCT 3'
VH5	5' GAGGTGAAAAAGCCCCGGGAGTCT 3'
VH6	5' CCTGTGCCATCTCCGGGACAGTG 3'
Fr1 con	5' AGGTGCAGCTG(G/C)(A/T)G(G/C)AGTC(G/A/T)GG 3'
Fr2	5' TGG(A/G)TCCG(A/C/G)CAG(G/C)C(T/C)CC(N)GG 3'
JH con	5' ACCTGAGGAAGACGGTGACCAGGGT 3'
JH3	5' TACCTGAAGAGACGGTGACCATTGT 3'
JH6	5' ACCTGAGGAGACGGTGACCCTGGT 3'

Results

In total, 127 bone marrow samples (containing a minimum of 10% plasma cells) were analysed by using the Fr3 PCR. Clonal rearrangements were demonstrated in 71 (56%). Single rearrangements predominated but biclonal rearrangements were seen in five cases. Rearrangements ranged from 87 to 173 bp in size (normal range 85–140 bp) with the vast majority (96%) lying within the normal fingerprint. This, in our experience, is in stark contrast to ALL where multiple rearrangements occur in

Table 2 VH gene usage

VH family	Multiple myeloma	Normal
VH1	11/57 (19%)	29%
VH2	6/57 (11%)	5%
VH3	28/57 (49%)	49%
VH4	9/57 (16%)	14%
VH5	3/57 (5%)	2%
VH6	0/57 (0%)	1%

A total of 57 clonal rearrangements were demonstrated in 52/98 cases analysed with the Fr1f PCR (biallelic rearrangements were seen in five of 52 positive cases). There is no obvious bias in the VH families used as has been reported for ALL and CLL.^{6,8,9}

Table 3 Summary of results obtained with the four IgH PCR strategies in 44 cases of multiple myeloma

PCR	JH con	JH mix
Fr3	26/44 (59%)	27/44 (61%)
Fr1f	26/44 (59%)	28/44 (63%)
Fr3 + Fr1f	34/44 (77%)	36/44 (82%)
Fr1 con	21/44 (48%)	22/44 (50%)
Fr2	21/44 (48%)	23/44 (52%)
Fr1 con + Fr2	28/44 (63%)	30/44 (68%)
Total	35/44 (80%)	37/44 (84%)

Each PCR technique was performed twice for each of the 44 cases using the JH consensus primer alone and an equimolar mixture of JH consensus, JH3 and JH6 primers (JH mix). Note that the Fr1 con and Fr2 PCR techniques have inferior detection rates compared with the Fr3 and Fr1f PCR techniques. The Fr2 PCR did, however, demonstrate a rearrangement in one case negative by both Fr3 and Fr1f. Clonal rearrangements were detected in two additional cases by using the JH mix; one case became positive by Fr3, Fr1f and Fr2 and the other by Fr1f, Fr1 con and Fr2.

40% with many lying outside the normal range (data not shown).

Diagnostic bone marrow specimens from 98 cases were also analysed with the Fr1f PCR, clonal rearrangements being demonstrable in 52 (53%) of 98 cases. Fr1f rearrangements ranged from 294 to 368 bp in size with 96% lying within the normal fingerprint (the normal range varies with each VH family). Biallelic rearrangements were seen in five cases. There did not seem to be any bias in VH gene usage (table 2) such that VH3 rearrangements predominated (49%) and VH5 and VH6 rearrangements were rare (5% and 0%, respectively).

A combination of both Fr3 and Fr1f PCR techniques demonstrated definitive clonal rearrangements in 92 (75%) of 122 cases (five cases were negative by Fr3 PCR and had insufficient DNA for analysis by Fr1f PCR). Fr1 con and Fr2 PCR techniques were also assessed in 44 cases of confirmed multiple myeloma (table 3). In these cases the Fr3 and Fr1f PCRs had demonstrated rearrangements in 26 (59%) cases with a combined yield of 34 (77%). Both the Fr1 con and the Fr2 techniques had inferior pick up rates, demonstrating clonal rearrangements in 21 (48%) of 44 cases with a combined yield of 28 (63%). The Fr2 PCR did, however, demonstrate a clonal rearrangement in one case which was negative by both Fr3 and Fr1f.

In six cases the clonal IgH rearrangement could only be demonstrated by Fr1f PCR. The Fr1 con PCR yielded rearrangements of 349–394 bp in size (normal range 350–405 bp) and the Fr2 rearrangements of 192–285 bp (normal range 240–285 bp). In both instances 98% of rearrangements lay within the normal fingerprint.

By utilising a mixture of JH primers (JH con, JH3 and JH6) rearrangements were demonstrated in a further two cases: one became positive by Fr3, Fr1f and Fr2 and the other by Fr1f, Fr1 con and Fr2.

Discussion

The sequence of clonal IgH rearrangements in multiple myeloma (unlike ALL) remain unchanged throughout the course of the disease,¹⁰ making it an ideal marker for studies of residual disease. PCR techniques which demonstrate such rearrangements either on the basis of their size or specific sequence are being increasingly used in the study of multiple myeloma. It has been possible with such techniques to demonstrate clonal rearrangements (identical with those seen in diagnostic bone marrow samples) in the peripheral blood of the majority of patients at diagnosis.^{11,12} Studies have also demonstrated clonal rearrangements in peripheral blood progenitor cell collections^{13,14}; and also that these may be eliminated by purging strategies such as CD34 selection or cell sorting.^{14–16} The presence of residual disease has also been assessed in patients who have undergone allogeneic¹⁷ and autologous¹⁸ transplantation.

It is clearly advantageous to be able to demonstrate IgH rearrangements in as many patients as possible. In this study we have compared the ability of the four commonly used PCR techniques to demonstrate clonal IgH rearrangements in multiple myeloma. We were able to demonstrate clonal rearrangements in 56% of cases with the Fr3 PCR and 53% with the Fr1f PCR. Barker *et al*¹⁹ were able to demonstrate clonal Fr1f rearrangements in 23/28 cases but this detection rate has not been achieved by others.^{5,7} However, by combining both techniques we were able to demonstrate definitive clonal rearrangements in 75% of cases. Recent reports have suggested that a greatly improved yield (up to 100% of cases) could be obtained with the Fr2 and Fr1 con PCR techniques.^{3–5} These studies involved small numbers of patients with multiple myeloma and we were unable to confirm their findings; both techniques detected clonal rearrangements in 21 (48%) of 44 cases with a combined yield of 28 (63%). We therefore recommend the routine use of both the Fr3 and Fr1f PCR techniques to demonstrate clonal IgH rearrangements in multiple myeloma. The Fr1 con and Fr2 PCR techniques have significantly inferior pick up rates and do not detect many additional rearrangements; the Fr2 PCR demonstrated one additional rearrangement in our series of 44 cases. Although the Fr1f technique requires six PCR reactions we believe it is important to use it as a significant number of rearrangements were only demonstrable by this technique. In addition, it has the added benefit of being 0.5–1 log more sensitive than the Fr3 PCR in the majority of cases (data not shown). It has also been suggested recently that some JH consensus primers may lack homology at their 3' ends with JH3 and JH6 segments.⁵ In this study we have assessed each PCR strategy with a JH consensus primer

alone and combined with JH3 and JH6 specific primers. By utilising the mixture of JH primers it was possible to demonstrate rearrangements in two additional cases; one became positive by Fr3, Fr1f and Fr2 and the other positive Fr1f, Fr1 con and Fr2.

It is clear therefore that it will not be possible to demonstrate clonal IgH rearrangements in approximately 20% of cases. This is likely to be due to the loss of VH primer binding sites secondary to somatic hypermutation which has been demonstrated by a number of investigators.^{10 20 21} The extent of mutation seems to be greater in multiple myeloma with a median of 8% of nucleotides mutated compared with 2% in CLL and 4% in follicular lymphoma.²² Extensive mutation has also been demonstrated in V_{kappa} genes, again the extent of mutation seemed to be greater in multiple myeloma than in other lymphoproliferative disorders.²³

In this study we were able to demonstrate clonal Fr1f rearrangements in 52 (53%) of 98 cases. There did not seem to be any bias in the VH genes used such that VH3 rearrangements predominated, and VH5 and VH6 rearrangements were rare. This pattern, which has been reported previously in a smaller series of patients,¹⁹ is also seen in follicular lymphoma²⁴ but not ALL and CLL where the over representation of VH5 and VH6 families is a well recognised phenomenon.^{6 8 9} In addition, certain specific VH genes—for example, VH4-21, VH4-18 and VH1 51P1, have been demonstrated to be used preferentially in ALL and CLL.²⁵⁻²⁷ Sequence data is more limited in multiple myeloma although it does seem that VH4-21 is not used preferentially.²⁸ We conclude that clonal IgH rearrangements can be demonstrated in the majority of patients with multiple myeloma. It would seem that the Fr3 and Fr1f PCR techniques provide the optimal combination demonstrating rearrangements in up to 80% of cases.

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