Assessment of IgH PCR strategies in multiple myeloma


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 PCR demonstrated clonal rearrangements in 26 (59%) cases with a combined yield of 54 (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.

Keywords: immunoglobulin heavy chain gene, myeloma, PCR.
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.

![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.](image)

### Table 1  Primer sequences used in each PCR strategy

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr3</td>
<td>5' CCGAGGACACGGGC/(C/T)/(C/G)TGATTTA CTG 3'</td>
</tr>
<tr>
<td>Fr1F V1</td>
<td>5' CCTCAGTGGAGGTCTCTCGAAGG 3'</td>
</tr>
<tr>
<td>VH2</td>
<td>5' GAGTAGCTGTCGTCGTCCTGAA 3'</td>
</tr>
<tr>
<td>VH3</td>
<td>5' GGTTCCTGAGACTCTCTGTCGCA 3'</td>
</tr>
<tr>
<td>VH4</td>
<td>5' TTGGA(G/A)ACTCTGTCCTCACC 3'</td>
</tr>
<tr>
<td>VH5</td>
<td>5' GAATGAAAAGCCCGGAGTCT 3'</td>
</tr>
<tr>
<td>VH6</td>
<td>5' CCGTGTGGCACTCTCCGGGAGAAT 3'</td>
</tr>
<tr>
<td>Fr1 con</td>
<td>5' AGTGACAGCTG/C/A/D(T)/CG.CGCGA/T/GTCG/A/T/GG 3'</td>
</tr>
<tr>
<td>Fr2</td>
<td>5' TGA(A/G)/TCCG/A/G/CAG(A/C)/CC(C/T/C)CC(N/G)GG 3'</td>
</tr>
<tr>
<td>JH con</td>
<td>5' ATGAGGAAGAAGGCTGACACGGGTGT 3'</td>
</tr>
<tr>
<td>JH3</td>
<td>5' ACTGAGGAAGAAGGCTGACACGGGTGT 3'</td>
</tr>
</tbody>
</table>

### 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 biconal 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
A total of 57 clonal rearrangements were demonstrated in 52/98 cases analysed with the Fr1f PCR (biallelic rearrangements were seen in five of 22 positive cases). There is no obvious bias in the VH families used as has been reported for ALL and CLL.12

Table 2  VH gene usage

<table>
<thead>
<tr>
<th>VH family</th>
<th>Multiple myeloma</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>11/57 (19%)</td>
<td>29%</td>
</tr>
<tr>
<td>VH2</td>
<td>6/57 (11%)</td>
<td>5%</td>
</tr>
<tr>
<td>VH3</td>
<td>28/57 (49%)</td>
<td>49%</td>
</tr>
<tr>
<td>VH4</td>
<td>9/57 (16%)</td>
<td>14%</td>
</tr>
<tr>
<td>VH5</td>
<td>3/57 (5%)</td>
<td>2%</td>
</tr>
<tr>
<td>VH6</td>
<td>0/57 (0%)</td>
<td>1%</td>
</tr>
</tbody>
</table>

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, 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 to use 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 collections11 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 autologous10 or allogeneic16 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 46% of cases with the Fr3 PCR and 53% with the Fr1f PCR. Barker et al9 were able to demonstrate clonal Fr1f rearrangements in 23/28 cases but this detection rate has not been achieved by others.3 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 4 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 Fr1f PCR may be superior to the Fr2 PCR to demonstrate residual disease, although we found that both techniques are 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.7 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 for Fr3, Fr1f and Fr2 and the other positive Fr1f, Fr1 con and Fr2.

It is clearly 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 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_{\text{appa}} 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,10 21 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.

This work was supported by the Leukaemia Research Fund and the Yorkshire Cancer Research Campaign.