Haematologica 1999; 84:328-335 original paper

Heteroduplex PCR analysis of rearranged immunoglobulin genes for clonality assessment in multiple myeloma

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ABSTRACT

Background and Objective. **Molecular analysis by PCR of monoclonally rearranged immunoglobulin (Ig) genes can be used for diagnosis in B-cell lymphoproliferative disorders (LPD), as well as for monitoring minimal residual disease (MRD) after treatment. This technique has the risk of false-positive results due to the *background* amplification of similar rearrangements derived from polyclonal B-cells. This problem can be resolved in advance by additional analyses that discern between polyclonal and monoclonal PCR products, such as the heteroduplex analysis. A second problem is that PCR frequently fails to amplify the junction regions, mainly due to somatic mutations frequently present in mature (post-follicular) B-cell lymphoproliferations. The use of additional targets (e.g. Ig light chain genes) can avoid this problem.**

Design and Methods. **We studied the specificity of heteroduplex PCR analysis of several Ig junction regions to detect monoclonal products in samples from 84 MM patients and 24 patients with B cell polyclonal disorders.**

Results. **Using two distinct VH consensus primers (FR3 and FR2) in combination with one JH primer, 79% of the MM displayed monoclonal products. The percentage of positive cases was increased by amplification of the V_λ-J_λ junction regions or κde rearrangements, using two or five pairs of consensus primers, respectively. After including these targets in the heteroduplex PCR analysis, 93% of MM cases displayed monoclonal products. None of the polyclonal samples analyzed resulted in monoclonal products. Dilution experiments showed that monoclonal rearrangements could be detected with a sensitivity of at least 10^{-2} in a background with >30% polyclonal B-cells, the sensitivity increasing up to 10^{-3} when the polyclonal background was < 1% of polyclonal B-cells.**

Interpretation and Conclusions. **Heteroduplex analysis of PCR amplified products is a simple and quick alternative for detecting monoclonally rearranged Ig genes in MM. This can be applied for diagnosis of B cell LPD and as a previous step in MRD strategies.**

Key words: myeloma, molecular diagnosis, rearrangements, heteroduplex, PCR

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B-cell lymphoproliferative disorders (LPD) can generally be diagnosed by routine cytomorphologic examination and/or by immunophenotyping.[1] The latter technique is based on expression of particular antigens, including the expression of surface and cytoplasmic immunoglobulins (Ig), which, in addition to cell lineage identification, can confirm the monoclonality of the cell proliferation based on Ig light chain restriction.[2,3] There are, however, some cases in which it is difficult to establish the monoclonality by immunophenotyping due to the absence of Ig expression, presence of oligoclonality, poor quality of the sample, or low numbers of tumor cells.[2] Tumor cells in B-cell LPD are derived from a single malignantly-transformed cell, with the implication that the Ig gene rearrangements of all malignant cells are identical.[3] The molecular analysis of such rearrangements can be used to prove or exclude the monoclonal nature of the proliferation. So far, this has generally been assessed by Southern blot (SB) analysis, which is a highly reliable but time-consuming and labor-intensive method.[3] This is the reason why PCR amplification of the junction regions of Ig genes is increasingly being investigated as an alternative to SB analysis.[4-9]

Although PCR techniques can be sensitive and fast, they have two major drawbacks in diagnostic clonality studies of Ig genes. Firstly, there are false-positive results due to the background generated by the amplification of rearranged Ig genes in polyclonal, reactive B-lymphocytes. Several methods have been used to solve these problems including direct sequencing of PCR products,[4,10-12] single-strand conformation polymorphism (SSCP),[13,14] denaturing gradient gel electrophoresis (DGGE),[15-18] temperature

gradient gel electrophoresis (TGGE),[19] gene scanning,[4,10,20,21] and heteroduplex analysis.[22-27] Among these techniques, heteroduplex analysis has proved to be a simple, fast, and cheap method for analysis of PCR products in the case of rearranged TCR genes.[22-25] The second problem is that rearrangements involving particular V gene segments might remain undetected when only consensus primers for this region are used. This is mainly due to the existence of primer mismatches within the FR3 and FR2 regions originating from somatic mutations, which occur frequently in mature post-follicular B-cell proliferations.[28-36]

In heteroduplex analysis, PCR products are denatured at high temperature and subsequently renatured to induce homo- or heteroduplex formation. Originally the heteroduplex technique was designed for mutation screening in genetic diseases,[37] but it has also been applied in clonality assessment, e.g. in T-cell lymphoproliferative disorders.[22-25] Heteroduplex analysis enables discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations, based on the presence of homoduplexes (PCR products with identical junction regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junction regions), respectively.[22-25]

As mentioned above, the heteroduplex technique has been used to assess monoclonality in T-cell proliferations, as well as in precursor B-cell acute and chronic lymphoid leukemias. The experience in mature (post-follicular) B cell LPD is, however, still limited, especially in MM.[26,27] MM is perhaps the best example of a monoclonal B cell disorder, since the monoclonal characteristic can be assessed not only at the molecular genetic level, but also in the protein product. In addition, its tumor cells frequently carry somatic mutations in the VDJ rearranged segments, which is the most frequent source of false negative results in monoclonal diagnosis by PCR amplification. Thus, it seems to be the perfect source of samples for standardization and evaluation purposes in monoclonal diagnosis. In the present paper we analyze the value of heteroduplex PCR analysis in bone marrow samples from MM patients as positive controls, using lymph nodes from reactive disorders as negative controls. In addition, the sensitivity of the heteroduplex analysis of Ig genes was also evaluated, as well as the quality of the PCR products to obtain the sequence of the CDR3 region, in order to assess its hypothetical applicability in MRD strategies.

Design and Methods

Cell samples

Cell samples were obtained from bone marrow aspirates of 84 MM patients and from lymph node biopsies of 24 patients with reactive disorders. The diagnosis of MM was made according to the criteria of the *Chronic-Leukemia-Myeloma-Task-Force*,[38] implying the presence of a monoclonal component in the urine and/or serum immunoelectrophoresis in all cases analyzed. PC count was determined based on strong positivity of the CD38 antigen and the presence of intracellular single Ig light chains (Igκ or Igλ).[39] The monoclonal serum component was IgG in 46 patients, IgA in 25, and IgD in 1. The remaining 12 patients had only monoclonal Ig light chains. The serum or urine monoclonal Ig light chain was κ in 64% and λ in 36%.

Samples from 24 reactive lymph node biopsies were used as controls of B-cell polyclonality with high numbers of polyclonal B-cells. The number of B-cells in the samples was determined by flow cytometry based on the cell surface expression of CD19 antigen, as reported elsewhere.[39] Those samples with less than 30% polyclonal B-cells were not included in the study. The polyclonality of the samples was confirmed through the absence of B cell disorders in the patients (clinical diagnosis and outcome) together with the absence of surface Ig light chain restriction.

DNA preparation and Southern blot analysis

Standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation[3,40] were used to isolate high molecular weight DNA. For Southern blot (SB) analysis, 10 µg of DNA were digested with the *Bgl*II and *Bam*HI/*Hind*III restriction enzymes, size fractionated and blotted to nylon membranes as described elsewhere.[3,41] The blots were hybridized with the ^{32}P-labeled IGHJ6 and IGKDE probes (DAKO, Carpinteria, CA, USA) and auto-radiographed for 72 hours.[42,43] All samples were analyzed by this method in order to confirm not only their B-cell monoclonality or polyclonality but also the presence of a sufficient number of clonal cells in the monoclonal samples.

PCR amplifications

In all samples, two PCR analyses of the VDJ region of the Ig heavy chain (IGH) gene were performed on 500 ng of genomic DNA. Two consensus V_H-J_H primer pairs were used for these amplifications: FR3-JHc and FR2-JHc (Table 1). PCRs were performed in a 50 µL final volume containing: 200 µmol/L of oligonucleotide primers, 200 mmol/L of each deoxynucleotide triphosphate, 2 mmol/L $MgCl_2$ and 1.5 U of Taq polymerase (Promega, Madison, WI, USA) in PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 1% Triton). Amplifications with the FR3-JHc primer pair were performed in a Perkin Elmer Cetus 9600 thermal cycler. The initial denaturation was of 5 minutes at 94ºC, followed by 35 cycles, consisting of 45" denaturation at 94ºC, 45" annealing at 55ºC and 45" extension at 72ºC, and a final extension at 72ºC for 10 minutes. When the FR2-JHc primer pair was used, the temperature conditions were denaturation at 94ºC for 60", annealing at 63ºC for 60" and extension at 72ºC for 60", with the same initial denaturation and final extension.

If no amplification was obtained, a third PCR was

Table 1. Primers and pairs used for the amplification of the Ig gene rearranged regions.

Gene	Name	Primer	Ref
IGH	VHc Fr3-5′	5′ CTGTCGACACGGCCGTGTATTACT 3′	10
	VHc Fr2-5′	5′ TGG(A/G)TCCG(C/A)CAG(G/C)C(T/C)¬(T/C)C(A/T/G/C)GG 3′	10
	JHc-3′	5′ AACTGCAGAGGAGACGGTGACC 3′	10
IGL	Vλc1-5′	5′ CAGTCTGTGCTGACTCAGCC 3′	*
	Vλc2-5′	5′ CAGTCTGTGCTGACTCAGCC(A/G)C 3′	*
	Jλc-3′	5′ TAGGACGGTCAGCTTGGTCCC 3′	*
IGK	Vκ I-5′	5′ GTAGGAGACAGAGTCACCATCACT 3′	26
	Vκ II-5′	5′ TGGAGAGCCGGCCTCCATCTC 3′	26
	Vκ III-5′	5′ GGGAAAGAGCCACCCTCTCCTG 3′	26
	Vκ IV-5′	5′ GGCGAGAGGGCCACCATCAAC 3′	26
	IgKRS-5′	5′ GTTATTCCCAAAAGCTCAATCTCAAAG 3′	26
	κ-del-3′	5′ CCCTTCATAGACCCTTCAGGCAC 3′	26

Primer pairs	Amplified fragment sizes
VHc Fr2 - JHc	240-300 bp
VHc Fr3 - JHc	80-150 bp
Vλc1 - Jlc	270-350 bp
Vλc2 - Jlc	270-350 bp
Vκ I - κ-del	420 bp
Vκ II -κ-del	450 bp
Vκ III - κ-del	450-470 bp
Vκ IV - κ-del	450 bp
IgKRS - κ-del	515 bp

**Tumkaya et al., manuscript in preparation.*

carried out in order to amplify the VJ junction regions of rearranged Ig λ light chain (IGL) gene. For this purpose, two PCRs were performed with the Vλ1-Jλc and Vλ2-Jλc primer pairs, as described by Tumkaya *et al.* (manuscript in preparation, Table 1). The final volumes and concentrations were the same as described above, with the exception of the final primer concentration, which was 100 nmol/L. The temperature conditions for amplification were as described.[26]

If still no amplification was obtained, a final trial was carried out using Ig κ light chain (IGκ) gene rearrangements of the κ-deleting element (κde) as target.[26] For this purpose, five PCRs per sample were carried out, using five primer pairs in which the oligonucleotide kde was used combined with the Vκ1, Vκ2, Vκ3, Vκ4 and IgκRS primers as described by Beishuizen *et al*.[26] The primer combinations and temperature program were as described.[26]

In order to test whether PCR products had been obtained after each amplification, 10 µL of the final reaction were analyzed by electrophoresis in a 2% Nu Sieve agarose gel (FMC, Rockland, ME, USA) in tris-borate electrophoresis buffer (TBE) and visualized under UV light after staining with ethidium bromide. In all experiments, negative (sterile distilled water) and positive controls were used.

Heteroduplex analysis

For heteroduplex analysis, PCR products were denatured at 94ºC for 5 min and subsequently cooled at several temperatures (40ºC, 20ºC and 4ºC) to induce duplex formation.[22-27] Briefly, the homo- and/or heteroduplexes were immediately loaded on a 6% non-denaturing polyacrylamide gel in 0.5 × Tris-Boric acid-EDTA (TBE) buffer, and run at room temperature. The visualization of products was made by silver nitrate staining (*fixation* for 6' in a 0.5% acetic acid, 10% ethanol solution, *staining* for 15' in a 0.1% $AgNO_3$ solution, *development* for 20' in a 0.15% formaldehyde, NaOH 1.5% solution, and *final fixation* for 10' in a 0.75% Na_2CO_3 solution). High DNA Mass-Ladder™ (Life Technologies, Inching, UK) was used as the DNA size marker.

Sensitivity studies

To determine the sensitivity of the heteroduplex PCR technique, several dilution experiments were performed. DNA from samples which yielded monoclonal products were serially diluted into two types of polyclonal DNA: 1) DNA extracted from polyclonal lymph nodes with more than 50% polyclonal B-cells, which were considered to be samples with a high polyclonal B-cell background, and 2) DNA extracted from the granulocyte phase of Ficoll separated cells from normal peripheral blood samples, with less than 1% of polyclonal B cells, which were considered to be samples with a low polyclonal B-cell background. These two types of serial dilutions subsequently underwent the same heteroduplex PCR analysis, and the sensitivity limit of the technique was considered to be the last dilution in which the homoduplex band was still clearly visible.

Results

Polyclonal samples

All samples from reactive lymph nodes lacked clonal Ig rearrangements by SB analysis. PCR analysis of the Ig gene junction regions produced no amplification or PCR products as a strong diffuse band in the agarose electrophoresis, which was difficult to distinguish from a real clonal band (Figure 1A). These problems were largely resolved after heteroduplex analysis and polyacrylamide gel electrophoresis, since all 24 polyclonal samples showed no product or a clearly distinguishable smear without any band at the position of homoduplexes (Figure 1B).

Multiple myeloma samples

Southern blot analysis

Upon SB analysis, all MM samples showed clonal IGH gene rearrangements (40 monoallelic and 44 biallelic), confirming the existence of B-cell monoclonality in all samples. κde was rearranged in 57% of the 84 cases (21% and 36% monoallelic and biallelic rearrangements, respectively). All cases with biallelic Kde rearrangements showed an Igλ monoclonal component in the serum or urine.

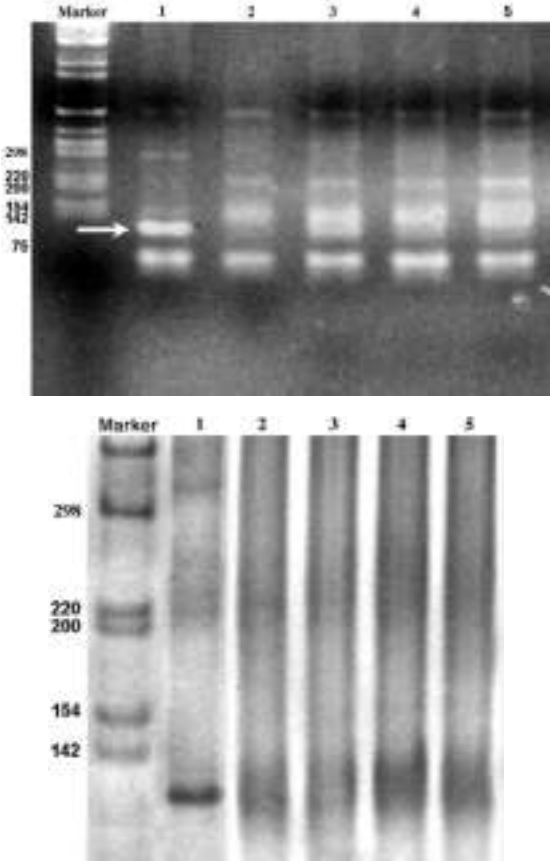

Figure 1. Comparison of results in polyclonal samples between direct agarose gel electrophoresis of PCR products and ethidium bromide staining (top) and polyacrylamide gel electrophoresis of PCR after heteroduplex formation and silver nitrate staining (bottom). Lanes 2 to 5, polyclonal samples. Lane 1: positive monoclonal control (arrows indicate the monoclonal band).

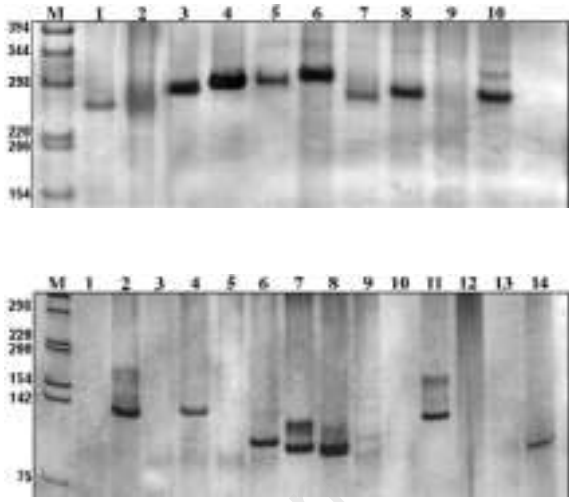

Figure 2. Heteroduplex PCR results in MM samples. A, amplification of VDJ_H using the FR2 and JHc primers. Positive monoclonal results are obtained in lanes 1 (positive control), 3, 4, 5, 6, 7, 8 and 10, while a smear is obtained in line 2 (negative control) and no amplification is obtained in lane 9. B, amplification of VDJ_H using the FR3 and JHc primers. Positive monoclonal results are obtained in lanes 2 (positive control), 4, 6, 7, 8, 11 and 14, while negative results are obtained in lanes 1, 3, 5, 9, 10, 12 and 13.

Monoclonality detection with heteroduplex PCR analysis

After heteroduplex PCR analysis using the primer pairs for the VDJH junction regions (FR2 and FR3 combined with JHc), 79% of cases (66 out of 84) showed monoclonal results (Figure 2). Regarding the value of the different primer pairs assayed, monoclonal results were obtained in 58% and 57% of cases when FR3 and FR2 were used, respectively. Positive results were obtained with both primers in 29 cases. In the remaining 37 cases, the monoclonal bands could be seen only with one of the two primer pairs.

In some cases, bands additional to the monoclonal homoduplex band could be seen. Such bands were always located at a size higher than 500 bp, and so did not complicate the interpretation of the clonal bands, which always had a size of 80-150 bp and 250-350 bp, in case of FR3 and FR2 primers, respectively.

PCR amplifications for the VJ_λ junction regions were carried out in the 18 samples in which no monoclonal bands were obtained for the VDJ_H region. Within these cases, monoclonal products were obtained in 10 (55%) samples. Accordingly, if we combine the amplification of VDJ_H and VJ_λ regions, monoclonal results were achieved in 90% (76 out of 84) of cases.

In the remaining 8 cases with previous negative results, PCR amplification of the junction region of IGκ-κde rearrangements by heteroduplex analysis resulted in positive results in two additional cases. Thus, employing the three junction targets, this methodology was able to produce monoclonal amplifications in 93% (78 out of 84) of the MM samples .

The six cases in which no monoclonal product was obtained had an Igκ monoclonal component in the serum, PC were positive for cytoplasmic Igκ by flow cytometry and no IGκ-κde rearrangements were detected by SB analysis.

Renaturation temperature and sensitivity

We also explored whether the renaturation strategy could influence clonality assessment. For this purpose, PCR amplifications of samples containing five different proportions of tumor DNA diluted in DNA with a high polyclonal B-cell background (50%, 25%, 10%, 5% and 0%) were denatured (94ºC, 5 minutes) and renatured at three different temperatures (40ºC, 20ºC and 4ºC). The renaturation temperature did not affect the identification of monoclonal bands when the FR3 primer was used (Figure 3), while clearer bands were seen at 4ºC with other primer sets.

Serial dilutions of monoclonal DNA diluted in DNA from highly polyclonal samples showed a sensitivity of

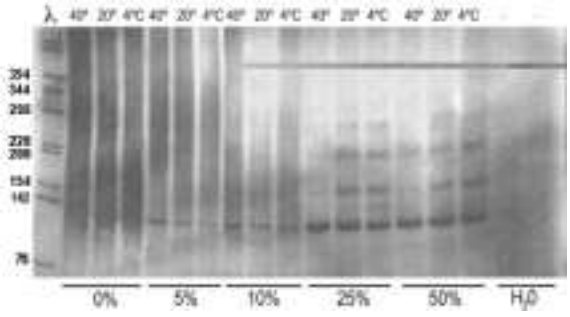

Figure 3. Dilution experiments with heteroduplex PCR analysis at different temperatures. The monoclonal bands can be seen at around 120 bp. No major differences can be seen between different renaturation temperatures (40, 20 and 4°C, indicated at the top of the figure).

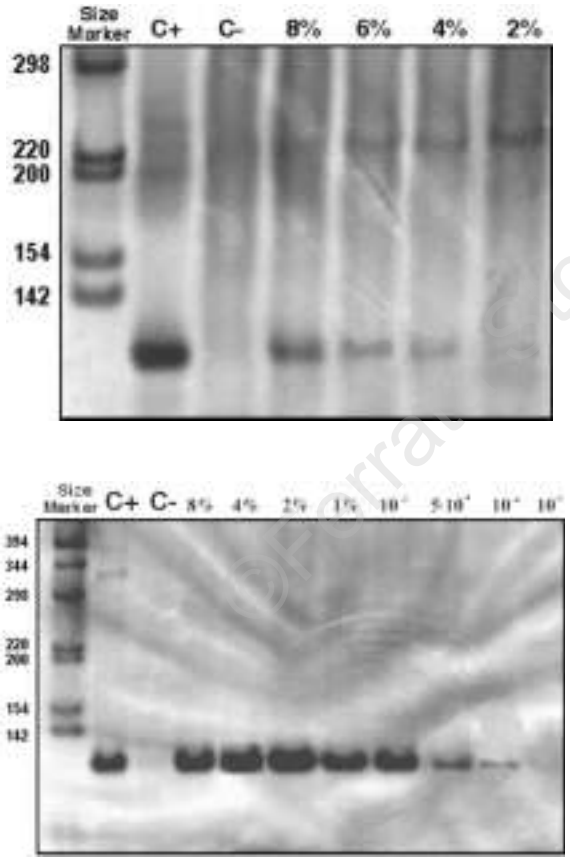

Figure 4. Dilution experiments of monoclonal samples. Top: DNA from a monoclonal sample diluted in DNA extracted from a sample with a high number of polyclonal B-cells. Bottom: DNA from a monoclonal sample diluted in DNA extracted from the granulocyte phase of Ficoll separated cells from a healthy donor (with <1% of polyclonal B-cells). A sensitivity of at least 4×10^{-2} and 5×10^{-4} could be achieved, respectively.

1 to 5×10^{-2} (identification of the monoclonal band when only 1% to 5% tumor cells were present in the sample) (Figure 4A). However, when the dilution experiments were done using dilutions of monoclonal DNA in DNA from samples with a low number of polyclonal B-cells (<1%), the sensitivity of the method reached at least 10^{-3} (Figure 4B). These experiments were repeated using cellular dilutions using a sample with 99% plasma cells and samples with polyclonal B cells or granulocytes; the results were comparable.

Discussion

Southern blot (SB) analysis of Ig and TCR genes has long been the only reliable method for molecular clonality assessment in the screening of lymphoproliferative disorders (LPD).[3,44] Since it is a long and labor-intensive method, several PCR based methods have been designed as alternatives over the last few years. These techniques, however, do not discriminate well between monoclonal and polyclonal PCR products. This problem can be resolved by further analysis of the PCR products, for example heteroduplex analysis. In this approach homo- and heteroduplexes are separated in non-denaturing polyacrylamide gels based on their tridimensional conformation. This approach does not require radioactive substrates or expensive equipment, and some reports have shown the validity of this strategy in T cell LPD,[22-25] as well as precursor B-ALL and B-CLL.[26-27] This technique can be more complicated in mature (post-follicular) B cell LPD due to the frequent existence of somatic mutations.[28-36] However, despite the fact that the information could be of clinical relevance in these entities, published information is still limited.

In this paper, we report on the use heteroduplex PCR analysis of junction regions from rearranged Ig genes to detect monoclonality in a typical post-follicular LPD, such as MM. This disease was chosen taking advantage of the facility of selecting standard samples for monoclonality and the high frequency of somatic mutations, which makes amplification of the rearranged regions difficult. The specificity of the method was demonstrated by analyzing 24 reactive lymph nodes. None of them showed amplification or polyclonal smears by heteroduplex analysis, although they had frequently yielded confusing (pseudo-clonal) bands in simple agarose electrophoresis. These can be interpreted as amplifications of rearranged regions of reactive B-lymphocytes in which agarose electrophoresis does not allow the distinction between polyclonal and monoclonal amplifications. Thus, heteroduplex analysis successfully eliminates the risk of false-positive results inherent to the PCR strategy for the diagnosis of monoclonal B cell LPD.

The second problem of PCR amplification of IGH junction regions with consensus primers, is the lack of amplification.[4,9,45] For instance, in the present

study, using the FR3 and FR2 consensus primers, amplification of the VDJ region failed in 42% and 43% of MM cases, respectively. The most probable cause of this problem is the existence of primer mismatches within the FR3 and FR2 regions due to the existence of somatic mutations in these regions, which are very frequent in mature (post) follicular LPD,[28-32] especially in MM.[33-36] However, this problem can be partially resolved by using several consensus primers or more than one PCR amplification target. Thus, upon combining the two FR consensus primers, positive amplification was obtained in 79% of cases. Moreover, this percentage increased up to 90% and 93% when amplifications of IGL and IGκ-κde junction regions were added. Combination of these approaches can thus be used for diagnostic and MRD purposes in the majority of B cell LPD. Higher percentages up to 100% can probably be reached via replacement of the Vλ-Jλ primer sets by Vκ-Jκ primer sets, because the latter can potentially detect clonality in the majority of Igκ+ LPD.

We also tested whether or not the temperature at which denatured fragments renature influences the heteroduplex results. In the initial descriptions of this technique, Langerak *et al.*[25] found that high renaturing temperatures could complicate the clonality diagnosis. In our experiments, similar results were obtained, except in the case of amplifications with the FR3/JHc primer set; with this primer pair, the temperature had a marginal effect. The reason for this result could be the size of the PCR product, which is quite small with this primer pair.

In some PCRs from our study, bands with a size greater than expected could be seen in the electrophoresis. Such bands did not, however, complicate the interpretation of the results. Their presence could be due to heteroduplex formation between two junction VDJ regions (biallelic rearrangement) or to the amplification of the VDJ junction region from the next JH segment where the JHc primer can also be annealed.

Analysis of serial dilutions of tumor DNA in DNA from samples with a high "background" of polyclonal B-cells revealed sensitivities of 1-5%, comparable to previous data from Langerak *et al*.[25] This sensitivity is enough for initial diagnosis, but it is not sufficient for detection of minimal residual disease (MRD), which requires higher sensitivities.[46,47] The sensitivity can, however, be increased up to levels of 10^{-3} when the "background" of polyclonal B-cells is virtually absent (<1%), which is the commonest situation in patients with MM and other B-cell lymphoproliferative disorders,[48,49] especially after intensive chemotherapy treatment.[50] Moreover, several approaches to MRD have shown that very high sensitivities might not be necessary from the clinical point of view, since highly sensitive methods can detect tumor cells (in theory) at very low levels in patients in long-term complete remission,[51] or even in normal individuals.[52,53] A typical example is the use of PCR for detection of the t(15;17) in acute promyelocytic leukemias, since those approaches with a relatively low level of sensitivity (10^{-3}-10^{-4}) are highly predictive from the clinical point of view.[54] In this way, some groups are exploring the value of methodologies with sensitivities around 10^{-4}, such as fingerprinting in the assessment of MRD in MM.[55]

This method also facilitates the identification of clonal sequences by direct sequencing of the PCR amplified junction regions of the Ig genes (data not shown). This alternative avoids the need for cloning, which usually complicates highly sensitive MRD strategies such as ASO-PCR, since they require tumor-specific probes or primers.[56-58]

We conclude that the heteroduplex PCR procedure of Ig genes employed here is a useful, simple, rapid, and cheap alternative to Southern blot analysis for the assessment of clonality in MM, and can be of value in some MRD strategies.

Contributions and Acknowledgments

RG-S and MG, were the main investigators and they designed the study. RG-S and RL-P carried out the PCRs of all samples, the literature revision and they wrote the paper. AWL and JJMVD were the inventors of the technique in its initial form, and the first PCRs were carried out in their laboratory. DG, MCC and AB performed all initial sample management and DNA extractions. MVM and IA were the clinicians responsible for the clinical management and clinical data acquisition. JFSM was the main coordinator of the group and reviewed the article to obtain the final form in which it was submitted. The order tries to take into account the time, work and scientific contribution given by all authors.

The authors thank Mark Anderson for his technical assistance.

Funding

This work has been partially supported by grants number 94/1564 and 96/1233 from the "Fondo de Investigación Sanitaria de la Seguridad Social" of the "Ministerio de Sanidad y Consumo", SPAIN, and the grant "Fundación Areces 1997".

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received October 5, 1998; accepted January 13, 1999.

References

1. Bennet JM, Catovsky D, Daniel M-T, et al. Proposals for the classification of chronic (mature) B and T lymphoid leukemias. J Clin Pathol 1989; 42:567.
2. Orfão A, Ruiz-Argüelles A, Lacombe F, Ault K, Basso G, Danova M. Flow cytometry: Its applications in hematology. Haematologica 1995; 80:69-81.

3. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991; 198:1-92.
4. Linke B, Bolz I, Pott C, Hiddemann W, Kneba M. Use of UITma DNA polymerase improves the PCR detection of rearranged immunoglobulin heavy chain CDR 3 junctions. Leukemia 1995; 9:2133-37.
5. Rockman SP. Determination of clonality in patients who present with diagnosis dilemmas: a laboratory experience and review of the literature. Leukemia 1997; 11:852-62.
6. Deane M, Mc Carthy KP, Wiedemann LM, Norton JD. An improved method for detection of B-lymphoid clonality by polymerase chain reaction. Leukemia 1991; 5:726-30.
7. Mc Carthy KP, Sloane JP, Wiedemann LM. Rapid method for distinguishing clonal from polyclonal B cell population in surgical biopsy specimens. J Clin Pathol 1990; 43:429-32.
8. Segal GH, Wittwer CT, Fishleder AJ, Stoler MH, Tubbs RR, Kjeldsberg CR. Identification of monoclonal B-cell populations by rapid cycle polymerase chain reaction. A practical screening method for detecting clonal rearrangements. Am J Pathol 1992; 141:1291-7.
9. Aubin J, Davi F, Nguyen-Salomon F, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia 1995; 9:471-9.
10. Kneba M, Bolz I, Linke B, Hiddemann W. Analysis of rearranged T-cell receptor β-chain genes by polymerase chain reaction (PCR) DNA sequencing and automated high resolution PCR fragment analysis. Blood 1995; 86:3930-7.
11. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junction diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993; 82:3063-74.
12. Van Oostveen JW, Breit TM, Borst J, et al. Polyclonal expansion of T-cell receptor γδ+ T-lymphocytes associated with neutropenia and thrombocytopenia. Leukemia 1992; 6:410-8.
13. Davis Th, Yockey CE, Balk SP. Detection of clonal immunoglobulin gene rearrangements by polymerase chain reaction and single-strand conformation polymorphism analysis. Am J Pathol 1993; 142:1841-7.
14. Koch OM, Volkenandt M, Göker E, et al. Molecular detection of clonal cell populations in acute lymphocytic leukemia by analysis of conformational polymorphisms of cRNA molecules of rearranged T-cell receptor γ and immunoglobulin heavy chain genes. Leukemia 1994; 8:946-52.
15. Bourguin A, Tung R, Galili N, Sklar J. Rapid, non radioactive detection of clonal T-cell receptor gene rearrangements in lymphoid neoplasms. Proc Natl Acad Sci USA 1990; 87:8536-40.
16. Grenier TC, Raffeld M, Lutz C, Dick LR, Jaffe EF. Analysis of T cell receptor-γ gene rearrangements by denaturing gradient gel electrophoresis of GC-clamped polymerase chain reaction products. Am J Pathol 1995; 146:46-55.
17. Theodoru I, Bigorgne C, Defaul MH, et al. VJ rearrangements of the TCRγ locus in peripheral T-cell lymphomas: analysis by polymerase chain reaction and denaturing gradient gel electrophoresis. J Pathol 1996; 178:303-10.
18. Wood GS, Tung RM, Haeffner AC, et al. Detection of clonal T cell receptor gamma gene rearrangements in early mycosis fungoides/Sézary syndrome by polymerase chain reaction as denaturing gradient gel electrophoresis /PCR/DGGE). J Invest Dermatol 1994, 103:34-41.
19. Linke B, Pyttlich J, Tiemann M, et al. Identification and structural analysis of rearranged immunoglobulin heavy chain genes in lymphomas and leukemias. Leukemia 1995; 9:840-7.
20. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995; 16:176-81.
21. Landman-Parker J, Aubin J, Delabesse E, et al. Simplified strategies for minimal residual disease detection in B-cell precursor acute lymphoblastic leukemia. Br J Haematol 1996; 95:281-90.
22. Bottaro M, Berti E, Biondi A, Migone N, Crosti L. Heteroduplex analysis of T-cell receptor γ gene rearrangements for diagnosis and monitoring of cutaneous T-cell lymphoma. Blood 1994; 83:3271-8.
23. Giachino C, Rocci MP, De Libero G, Ordea G, Ansaldi N. An alternative approach to the assessment of γδ T-cell clonality in celiac disease intestinal lesions though cDNA heteroduplex analysis of T-cell receptor VJ junctions. Hum Immunol 1994; 40:303-11.
24. Sottini A, Quiros-Roldan E, Albertini A, Primi D, Imberti L. Assessment of T-cell receptor β-chain diversity by heteroduplex analysis. Hum Immunol 1996; 48:12-22.
25. Langerak AW, Szczepanski T, Van der Burg M, Wolvers-Tettero ILM, Van Dongen JJM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia 1997; 11:2192-9.
26. Beishuizen A, de Bruijn MA, Pongers-Willemse MJ, et al. Heterogeneity in junction regions of immunoglobulin κ deleting element rearrangements in B cell leukemias: a new molecular target for detection of minimal residual disease. Leukemia 1997; 11:2200-7.
27. Szczepanski T, Langerak AW, Wolvers-Tettero IL, et al. Immunoglobulin and T cell receptor gene rearrangement patterns in acute lymphoblastic leukemia are less mature in adults than in children: implications for selection of PCR targets for detection of minimal residual disease. Leukemia 1998; 12:1081-8.
28. Du MQ, Diss TC, Xu CF, Wotherspoon AC, Isaacson PG, Pan LX. Ongoing immunoglobulin gene mutations in mantle cell lymphomas. Br J Haematol 1997; 196:124-31.
29. Matolcsy A, Warnke RA, Knowles DM. Somatic mutations of the translocated bcl-2 gene are associated with morphologic transformation of follicular lymphoma to diffuse large-cell lymphoma. Ann Oncol 1997; 8(Suppl 2):119-22.
30. Matolcsy A, Casali P, Warnke RA, Knowles DM. Morphologic transformation of follicular lymphoma is associated with somatic mutation of the translocated Bcl-2 gene. Blood 1996; 88:3937-44.
31. Tamaru J, Hummel M, Marafioti T, et al. Burkitt's lymphomas express VH genes with a moderate number of antigen-selected somatic mutations. Am J Pathol 1995; 147:1398-407.
32. Zelenetz AD, Chen TT, Levy R. Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection. J Exp Med 1992; 176:1137-48.
33. Bakkus MHC, Hermain C, Van Riet B, Van Camp B, Thielemans K. Evidence that multiple myeloma Ig chain VDJ genes contain somatic mutations but show no intraclonal variation. Blood 1992; 9:2326-35.
34. Kosmas C, Viniou N-A, Stamatopoulos K, et al. Analysis of the κ light chain variable region in multiple myeloma. Br J Haematol 1996; 94:306-14.
35. Kiyoi H, Naito K, Ohno R, Naoe T. Comparable gene structure of the immunoglobulin heavy chain variable region between multiple myeloma and normal bone marrow lymphocytes. Leukemia 1996; 10:1804-12.

36. Hallek M, Leif Bergsagel P, Anderson KC. Multiple myeloma: increasing for a multistep transformation process. Blood 1998; 91:3-21.
37. White MB, Carvalho M, Derse D, O'Brien SJ, Dean M. Detecting single base substitutions as heteroduplex polymorphisms. Genomics 1992; 12:301-6.
38. Chronic Leukemia-Myeloma Task Force, National Cancer Institute. Proposed Guidelines for protocol studies. II. Plasma cell myeloma. Cancer Chemother Rep 1973; 4:145-58.
39. Orfão A, García-Sanz R, López-Berges MC, Vidriales MB, González M, San Miguel JF. A new method for the analysis of plasma cell DNA content in multiple myeloma samples using a CD38/propidium iodide double staining technique. Cytometry 1994; 17:332-9.
40. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
41. García-Sanz R, Vargas M, González M, et al. Detection of single and associated lesions on the Bcl-1, Bcl-2, Bcl-6, c-Myc, p53, and p16 genes in B-cell non Hodgkin's lymphomas. Value of molecular analysis for a better assignment of the histologic subtype. Haematologica 1998; 83:209-16.
42. Beishuizen A, Verhoeven M-AJ, Mol EJ, Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Detection of immunoglobulin heavy-chain gene rearrangements by southern blot analysis: recommendations for optimal results. Leukemia 1993; 7:2045-53.
43. Beishuizen A, Verhoeven MA, Mol EJ, van Dongen JJM. Detection of immunoglobulin κ light-chain gene rearrangement patterns by Southern blot analysis. Leukemia 1994; 8:2228-36.
44. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. Clin Chim Acta 1991; 191:93-174.
45. Segal GH, Jorgensen T, Aneal S, Masih MD, Braylan RC. Optimal primer selection for clonality assessment by polymerase chain reaction analysis: I. Low grade B-cell lymphoproliferative disorders of nonfollicular center cell type. Hum Pathol 1994; 12:1269-75.
46. Van Dongen JJM, Szcepanski T, de Bruijn MAC, et al. Detection of minimal residual disease in acute leukemia patients. Cytokines Molecul Ther 1996; 2:121-33.
47. Van Dongen JJM, San Miguel JF. Techniques for detection of minimal residual disease in leukemia patients. Meet the Expert Sessions of the Second EHA, Paris, 1996.
48. Pilarski LM, Mant MJ, Reuther BA, Belch A. Severe deficiency of B lymphocytes in peripheral blood from multiple myeloma patients. J Clin Invest 1984; 74: 1301-6.
49. Monserrat E, Bosch F, Rozman C. B-cell chronic lymphocytic leukemia: recent progress in biology, diagnosis and therapy. Ann Oncol 1997; 8:93-101.
50. San Miguel JF, Hernandez MD, González M, et al. A randomized study comparing the effect of GM-GSF and G-GSF on immune reconstitution after autologous bone marrow transplantation. Br J Haematol 1996; 94:140-7.
51. Price CGA, Meerabux J, Murtagh S, et al. The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 1991; 9:1527-32.
52. Liu Y, Hernández AM, Shibata D, Cortopassi GA. BCL2 translocation frequency rises with age in humans. Proc Natl Acad Sci USA 1994; 91:8910-4.
53. Limpens J, Stad R, Vos C, et al. Lymphoma-associated translocation t(14;18) in blood B cells in normal individuals. Blood 1995; 85:2528-36.
54. Diverio D, Pandolfi PP, Rossi V, Biondi A, Pelicci PG, Lo Coco F. Monitoring of treatment in acute promyelocytic leukemia by RT-PCR. Leukemia 1994; 7:1105-7.
55. Bird JM, Bloxham D, Samson D, et al. Molecular detection of clonally rearranged cells in peripheral blood progenitor cell harvests from multiple myeloma patients. Br J Haematol 1994; 88:110-6.
56. Billadeu D, Anam L, Thomas W, et al. Detection and quantification of malignant cells in the peripheral blood of multiple myeloma patients. Blood 1992; 80:1818-24.
57. Corradini P, Voena C, Astolfi M, et al. High-dose sequential chemoradiotherapy in multiple myeloma: residual tumor cells are detectable in bone marrow and peripheral blood cell harvests and after autografting. Blood 1995; 85:1596-602.
58. Vescio RA, Han EJ, Schiller GJ, et al. Quantitative comparison of multiple myeloma tumor contamination in bone marrow harvest and leukapheresis autografts. Bone Marrow Transplant 1996; 18:103-10.