Genotypic Analysis in Primary Cutaneous Lymphomas Using the Standardized Biomed-2 Polymerase Chain Reaction Protocols

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Abstract. The European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936 has defined standardized protocols for polymerase chain reaction (PCR) amplification of different loci of the T-cell receptor (TCR) and immunoglobulin (Ig) genes with a view to achieving greater sensitivity and specificity in the assessment of clonality of lymphoid neoplasms. To assess T-cell clonality, analysis of TCRβ gene and TCRγ rearrangements (useful in cases of Tγδ+ cell neoplasms) is proposed alongside that of TCRγ. For analysis of B-cell clonality, along with the framework (FR) III segment of the IgH gene, other segments are studied (FRI, FRII) in addition to Igλ and Igκ genes or incomplete DJ rearrangements of the IgH gene and the κ deleting element. The results of the amplification are read using automatic reading systems (GeneScan) or using a heteroduplex system.

Key words: cutaneous lymphoma, gene rearrangement, clonality, polymerase chain reaction, BIOMED-2, GeneScan.

Introduction

In recent years, molecular biology and pathology laboratories have introduced a set of molecular biology techniques based on polymerase chain reaction (PCR) into their diagnostic procedures. The aim of these techniques is to demonstrate the presence of a dominant proliferation of a monoclonal T- or B-cell population in atypical cutaneous lymphoid infiltrates (genotyping). 1-3 The results of genotyping, when interpreted in the appropriate clinical and pathological context, represent an important diagnostic tool in lymphoid malignancies (lymphomas), particularly in those cases in which the morphologic findings and immunophenotyping are inconclusive. 4,5

PCR techniques can be used not only with fresh or frozen samples, but also with archival paraffin-embedded samples. The only requisite is that relatively small DNA fragments are conserved (>100-200 base pairs [bp]). 1 This is doubtlessly an advantage over other techniques, such as Southern blotting, which requires large DNA fragments (>1000 bp)
and which is therefore limited to the study of fresh or frozen material.\textsuperscript{6,7} PCR-based techniques avoid the need for exposure to radioactive agents, produce results more rapidly, and require less work in the laboratory. The differences in methodology between laboratories lie mainly in the choice of oligonucleotide primers and in systems for reading or analyzing the amplified PCR fragments.\textsuperscript{2,8-14}

The European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936 was designed with a view to developing standardized PCR protocols for studying B- and T-cell clonality for diagnostic purposes in lymphoproliferative processes. It defines different protocols using multiple primers for different loci of T- and B-cell receptor genes in an attempt to increase the sensitivity and specificity of detection of T- and B-cell clonality.\textsuperscript{15,16}

**T-Cell Receptor/Immunoglobulin H Gene Rearrangement**

The B-cell receptor is an immunoglobulin (Ig)—specific to each cell—that is formed from 2 heavy chains (κ chains) and 2 light chains (λ chains). The T-cell receptor (TCR) is formed by joining an α subunit to a β subunit (α/β phenotype) or a γ subunit to a δ subunit (γ/δ phenotype). The genes that encode Ig and TCR proteins have different variable (V), diversity (D), and joining (J) segments that undergo rearrangement during the initial phases of lymphoid cell differentiation.\textsuperscript{7,18}

This process of gene rearrangement is initiated by joining one of the possible D segments to one of the possible J segments. In the case of IgH, TCRβ, and TCRδ genes, one of the V segments is subsequently joined (Figure 1). In the case of Igλ, Igκ, TCRα, and TCRγ genes, V and J segments are joined directly (Figure 2). This rearrangement follows a hierarchical order. During differentiation into a B cell, the IgH gene is rearranged first, followed by the Igκ gene, which is subsequently deleted to form an Igλ+ cell. During differentiation into a T cell, first the TCRδ gene undergoes rearrangement, followed by the TCRγ gene, then the TCRβ gene, with subsequent deletion of the TCRδ gene, and finally the TCRα gene is rearranged. The functional rearrangement of the Ig and TCR genes generates the expression of different receptors (Ig, TCRγδ, or TCRαβ) on the cell surface. These TCRγδ or TCRαβ configurations appear in the post-thymic T cells, with the TCRγδ configuration corresponding to the minor fraction.\textsuperscript{18}

The IgH gene (chromosome 14q32.3) has between 46 and 52 functional V\textsubscript{H} segments depending on each individual’s haplotype. These haplotypes can be grouped into 6 or 7 subgroups or families according to homology. In addition, there are 27 functional D\textsubscript{H} segments grouped into 7 families (D\textsubscript{H}1-7) and 6 functional J\textsubscript{H} segments. The Igκ gene (chromosome 2p11.2) contains multipleVk segments.

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**Figure 1.** VDJ rearrangement of the IgH gene. The V regions of the IgH gene are formed from 3 hypervariable or complementary determining regions (CDR) I, CDR II, and CDR III. These CDR segments alternate with 4 segments of little variability denoted framework regions (FR), where hybridization of the PCR primers occurs (arrows).
grouped into 7 families and 5 functional JH segments. The Igλ gene (chromosome 22q11.2) has 73 or 74 Vλ segments grouped into 11 families and 7 Jλ segments (4 functional: Jλ1, Jλ2, Jλ3, and Jλ7) that encode the 4 Igλ isotypes.17,19

The TCRγ locus (chromosome 7p14) has 14 variable Vγ gene segments (10 functional or combinatorial); 5 Jγ joining segments belonging to 1 of 2 groups, Jγ1 (Jγp1, Jγp, and Jγ1) and Jγ2 (Jγp2 and Jγ2); and 2 constant regions Cγ1 and Cγ2. The Vγ segments are separated into 4 subgroups according to their homologous sequence: group I (Vγ1-8) has 5 functional segments and 4 pseudogenes; groups II, III, and IV are formed from single segments, Vγ9, Vγ10, and Vγ11, respectively. The TCRγ gene (chromosome 7q34) is formed by rearrangement of 1 of the 65 Vβ segments (39-47 of which are functional and grouped according to homology into 23 families) with 1 of the Jβ segments (2 families, Jβ1-2).18

The combinatorial nature of V, D, and J segments gives rise to a repertoire of $2 \times 10^6$ possible combinations in the case of Ig, 3 $\times 10^9$ possible rearrangements for TCRβ, and 5 $\times 10^9$ TCRγ6 molecules. The diversity of these possible combinations is increased through insertion or deletion of nucleotides at segment joining regions, giving rise to more than $10^{12}$ possible combinations in the total repertoire of Ig and TCR molecules. Finally, in the case of B cells, the variability can also be increased through somatic hypermutations in the V segment of the IgH gene and the light chains of the mature B cells on their transit through the germinat center of lymphoid follicles, thereby further increasing the diversity and antigen specificity.17-19

Technical Aspects of the BIOMED-2 Project

DNA Quality Control

A crucial aspect in the application of the BIOMED-2 program is DNA quality control, which is particularly relevant in the case of paraffin-embedded samples. It is recommended to run a PCR to amplify control genes with 5 primer pairs for amplification of 100, 200, 300, 400, and 600 bp fragments. This gives an indication of the quality of the DNA in the sample analyzed. All PCR fragments in the BIOMED-2 project have fewer than 600 bp (Figure 3). PCR should be done with 2 different amounts of DNA to exclude the effect of PCR inhibitors (Table 1).15

Fresh or frozen tissue is the best for obtaining good quality DNA; however, in routine practice, most samples studied will be formalin-fixed and embedded in paraffin. The DNA obtained from such samples is usually of poor quality, particularly when the sample is more than 2 years old. The fixation procedure used and the quality of the tissue studied are also important factors,20 and so it is essential to run a control of DNA quality before amplification. If, in the quality control procedure, the DNA obtained is not longer than the gene or segment amplified, the results obtained are usually meaningless. It is also advisable to perform histologic control at the same time as obtaining tissue for DNA extraction to ensure that the sample studied is representative.

Analysis of PCR Fragments

Recently, automated reading systems have been developed for amplifying PCR fragments; these afford greater discriminatory power than traditional electrophoresis-gel-based reading systems.21-28 The BIOMED-2 project recommends reading PCR amplified products by capillary electrophoresis with fluorochrome-labeled primers (GeneScan) or using polyacrylamide gels for heteroduplex analysis.29-31

As all the cells of a lymphoid malignancy originate from a malignant clone, their DNA will have undergone the same rearrangement, and so on studying VDJ rearrangement by PCR, 1 or 2 PCR products will be obtained (rearrangement of 1 or 2 alleles). In contrast, if the DNA...
is derived from a polyclonal population, many VDJ rearrangements will be present. Visualizing the PCR product can be done by acrylamide gel electrophoresis or capillary electrophoresis. With gel electrophoresis, many discrete bands will be seen in the case of a polyclonal population or 1 or 2 bands in the case of a monoclonal population. Capillary electrophoresis reveals multiple peaks with a normal distribution of molecular weights for polyclonal populations and just 1 or 2 peaks for monoclonal ones.

Heteroduplex analysis uses double-stranded PCR products which are separated according to their length and conformation, whereas GeneScan analyzes single-stranded products which are separated only according to their length. In the case of a sample with a monoclonal lymphoid population, a variable proportion of homoduplexes will be formed. Visualization by polyacrylamide gel electrophoresis reveals a band corresponding to an appropriate length. Samples containing a high proportion of polyclonal cells produce abundant heteroduplexes with little likelihood of homoduplex binding.

GeneScan can distinguish between amplified products that differ by a single bp, thereby allowing the detection of the same clone in different samples or tissues without the need for sequencing. The heteroduplex analysis is considered to have greater sensitivity and specificity than the GeneScan system, particularly when amplifying Igκ, κδ, and Igλ rearrangements. In the analysis of DJ segments of the IgH gene, heteroduplex analysis or GeneScan can be used interchangeably.15

Fluorescent Fragment Analysis (GeneScan)

In the GeneScan system, 1 of the primers is labeled with fluorochrome. After amplification, the PCR products are discriminated or separated in a polymer capillary electrophoresis system in a process that involves mixing 1 µL of PCR product with 0.5 µL of a standard molecular weight product (GeneScan 400–ROX, Applied Biosystems, Foster City, CA, USA) in 9 µL of deionized formamide for denaturing into single DNA strands. The products are detected automatically by fluorescence reading with a laser system in an automatic DNA sequencer (ABI Prism 3100, Applied Biosystems). A result is considered monoclonal if 1 or 2 peaks of amplified product with an appropriate bp length for the PCR in question are obtained. Detection of 3 to 5 peaks is considered an oligoclonal result, and more than 5 peaks of different lengths would be interpreted as a polyclonal result. To consider a result as a truly monoclonal, the height of the peak should be at least twice that of the normal distribution of the polyclonal peaks, which correspond to the presence of reactive cells in the infiltrate (Figure 4).

The overall sensitivity for detection of B- or T-cell clonality of the BIOMED-2 protocols which use GeneScan for reading is approximately 0.5%-5%. The limits of detection depend on the technique used, the quality of the sample (paraffin-embedded samples have a lower yield), and the proportion of reactive lymphoid cells present in the sample. The technique is considered sufficiently sensitive with detection of a clonal population that comprises 5%-10% of the total lymphoid population of the infiltrate.

Heteroduplex Analysis

The PCR products are denatured by heating (95°C for 5 minutes) followed by rapid renaturation at low temperature (4°C for 1 hour) in an attempt to facilitate renewed spontaneous and, in this case, random hybridization in such a way that homoduplex or heteroduplex DNA fragments are obtained with a different conformation. In the case of heteroduplexes, the different conformations obtained give rise to different physical
characteristics, and so they can be separated by means of nondenaturing polyacrylamide gel electrophoresis. Homoduplexes achieve a perfect hybridization and migrate more quickly. The PCR products corresponding to monoclonal samples form a high proportion of homoduplexes that are mixed with heteroduplexes obtained from other polyclonal or reactive lymphocytes present in the sample and these are clearly separated into bands in the polyacrylamide gel. The polyclonal samples only give rise to heteroduplexes (Figure 5).

Heteroduplex analysis is rapid and simple, and has an overall sensitivity for the detection of B- or T-cell clonality of approximately 1%-10% (using the BIOMED-2 protocols), even when archival paraffin-embedded samples are studied.

Sensitivity and Specificity

**False Negatives (Sensitivity)**

Failure to detect clonality may occur when the molecular weight of the fragment to be amplified is greater than that indicated by the DNA quality control PCR. The use of multiple primers designed for the BIOMED-2 project for different segments can minimize the number of false negatives that arise from hybridization errors due to lack of specificity or homology with the primers, particularly when studying B-cell clonality, in which somatic hypermutations in the V<sub>H</sub> segment can cause hybridization failure. 19

**False Positives (Specificity)**

Detection of false positives, or pseudoclonality, becomes particularly important in cases in which the lymphoid infiltrate has reactive characteristics, with scant cellularity, or when small samples are studied. 20 This is a crucial element in the BIOMED-2 protocols, and to minimize this phenomenon, it is recommended to carry out the analysis in duplicate and, if possible, to study more than one sample from the same patient. Some authors recommend that this particular type of sample should be analyzed simultaneously using GeneScan and heteroduplex analysis, given the higher rate of false positives for GeneScan in such situations.

If the same clonality peak cannot be reproduced, the results should be considered as negative or pseudoclonal. It is also recommended to check in duplicate the clonality of a lymphoproliferative process by detecting a dominant clone using different Ig and TCR targets or gene segments. Finally, it should be remembered that detection of a monoclonal rearrangement of the TCR or Ig gene cannot unequivocally be interpreted as marker of a specific cell line or population. If the exceptional case of a composite lymphoma is ruled out, double aberrant T- and B-cell

### Table 1. BIOMED-2 Polymerase Chain Reaction (PCR) Protocols

<table>
<thead>
<tr>
<th>PCR Reagents</th>
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<tr>
<td>100 ng DNA</td>
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<tr>
<td>10 pmol of each primer (fluorochrome labeled or not depending on the reading system used). The number and type of primer for each tube depends on each specific PCR.</td>
<td></td>
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<tr>
<td>Nucleotides for a final concentration of 200 µM</td>
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<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt; at a final concentration of 1.5 µM</td>
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<tr>
<td>1-2 units of Taq polymerase (AmpliTaq Gold [Applied Biosystems, Foster City, CA, USA], depending on whether or not the PCR tube has more or less primers</td>
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<tr>
<td>ABI Buffer II or ABI Gold (AmpliTaq Gold [Applied Biosystems]) for a final volume of 50 µL</td>
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</table>

| PCR Conditions |  |
| Preactivation of Taq polymerase for 7 min at 95°C |  |
| Hybridization temperature: 60°C |  |
| Number of cycles: 35 |  |
| Cycle phases: |  |
| Denaturing: 30 (to 45) s (new PCR systems are faster) |  |
| Hybridization: 30 (to 45) |  |
| Extension: 30 s (to 1.5 min) |  |
| Final 10 min extension |  |
| Product storage possible at room temperature or at 15°C |  |
rearrangements or cross rearrangements (cross lineage) may be detected in the same sample, particularly in immature cell malignancies, although this can also be seen in 5%-10% of mature cell neoplasms.

**Study of T-Lymphoid Clonality**

**Technical and Methodological Aspects**

Amplification of the VJ gene of the TCRγ subunit by PCR is the most widely used method to evaluate clonality of an infiltrate when a T-cell origin is suspected. The technique is relatively simple, given that the gene rearrangement involves a limited repertoire of gene segments. The length of the amplified segment (approximately 200 bp) can yield results in most archival tissue samples. The TCRγ gene is rearranged in most T cells irrespective of whether they express TCRαβ or TCRγδ on their surface.

PCR analysis of the complete VDJ gene or incomplete DJ gene that encodes the TCRβ subunit is a more complex technique. The TCRβ subunit has a greater number of combinatorial possibilities, so more primers are needed, and the amplifiable segments are much longer or heavier (Figure 6). The TCRβ gene is rearranged not only in all TCRαβ cells but also in many TCRγδ ones. PCR analysis of the TCRβ gene is a complementary test that should be done when there is reasonable suspicion of T-cell lymphoma and PCR-TCRγ results are inconclusive. By combining such studies, T-cell clonality can be detected in almost all samples of T-cell lymphoma.

Likewise, TCRδ gene rearrangements can be detected. This is useful both in the study of immature lymphoid malignancies and in lymphoproliferative processes arising from TCRγδ cells. As a result of its complexity (due to the high number of combinatorial possibilities with segments widely spaced on the genome), the TCRα gene is not included in the BIOMED-2 project.

**Results for Cutaneous T-Cell Lymphoma**

Most studies that use electrophoresis gels detect clonality in between 40% and 90% of the samples of cutaneous T-cell lymphoma (CTCL). Different technical aspects, type
of reading system of the amplified products, and sample characteristics (paraffin-embedded or frozen, differing proportions of early and advanced stages) might explain the differences between the different series. Monoclonal lymphoid proliferation is usually detected more often in advanced stages of mycosis fungoides and in Sézary syndrome (90%-100%) compared to early mycosis fungoides lesions (50%-70%).

With application of PCR techniques and highly sensitive and specific reading systems (GeneScan, heteroduplex analysis), lymphoid monoclonality can be detected in approximately 60%-70% of CTCL samples. In the series reported by Ponti and coworkers, 162 of the 194 samples of CTCL (83.4%) were found to be clonal using PCR amplification of TCRγ and heteroduplex analysis. Similar results were obtained by the same authors using the GeneScan system (84%).

In our experience, half of the samples of lymphomatoid papulosis from frozen samples are clonal, as well as two-

Figure 5. Heteroduplex analysis. Heteroduplex analysis can be more informative than GeneScan for certain rearrangements such as in the case of Igλ, in the figure.

A dominant clone can be detected. Unlike most studies, Cherny et al only detected 42% of clonal samples with heteroduplex analysis in a series of CTCL; however, this result does not appear to be representative because of the small number of cases analyzed (n=7).

Application of the BIOMED-2 protocols with the GeneScan system has achieved high sensitivity in the study of different cutaneous T-cell lymphoproliferative processes. Although results are lacking in large series with paraffin-embedded samples, T-cell clones have been detected in between 63% and 85% of cutaneous samples of mycosis fungoides/Sézary syndrome by PCR with GeneScan of TCRγ and in between 63% and 75% by PCR with GeneScan of TCRβ.

A dominant clone is detected slightly less often in CD30+ lymphoproliferative processes than in other CTCL, perhaps because of the specific characteristics of a lymphomatous infiltrate that usually has a heterogenous cell population. In our experience, half of the samples of lymphomatoid papulosis from frozen samples are clonal, as well as two-

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Figure 5.
thirds of CTCL CD30+ samples. In a recent study of paraffin-embedded samples of lymphomatoid papulosis and CD30+ CTCL by PCR with GeneScan, a T-cell clone was detected in only 22% of the cases of lymphomatoid papulosis (4/18), particularly in those forms with a high proportion of atypical cells. In that study, the detection rate was 65% for CD30+ CTCL samples.

Those results indicate that an important feature of CTCL diagnosis is demonstrating a dominant clone given that the presence of a polyclonal rearrangement pattern is uncommon. However, the presence of a T-lymphocyte clone has also been detected in a variable number of samples from chronic inflammatory dermatoses that were considered benign but that showed an uncertain relationship with subsequent development of actual CTCL. Some authors have put forward the concept of "cutaneous lymphoid dyscrasias" to denote this group of processes. Lichenoid pityriasis, certain cases of pigmented purpuric dermatoses, atypical lobular panniculitis, certain drug reactions, and even a group of entities considered as "parapsoriasis" would be included in such a group.

The differential diagnosis for parapsoriatic lesions, defined as persistent erythematous-desquamative plaques without histopathologic criteria for mycosis fungoides, with respect to early mycosis fungoides lesions can pose substantial practical difficulties. There is certain controversy concerning the rate of detection of T-cell clonality in such cases. Some authors detect T-lymphocyte clonality in almost 50% of the samples, and suggest that these cases be considered as premycosis fungoides lesions or truly incipient mycosis fungoides lesions. However, other authors almost never detect clonality. The reason for these discrepancies is probably the lack of standard criteria for defining the concept of parapsoriasis (Table 2). In our experience, application of PCR for TCRγ can detect clonality in parapsoriatic lesions in a very few cases—less than 5%—whereas approximately 70% of the cases of mycosis fungoides in early stages are found to be clonal.

Finally, GeneScan reading can be used to assess clonality agreement between samples of different lymphoid processes that occur simultaneously or successively in the same patient (for example, mycosis fungoides and lymphomatoid papulosis lesions). If it is the same clone that is being detected, the monoclonal peak should be identical and correspond to the same length; this finding obviates the need to sequence the PCR products.

Data published on detection rates of a dominant clone in reactive T-lymphocyte proliferative processes is very variable (0%-24%), and comes mainly from studies that use traditional electrophoresis systems. Ponti and coworkers were able to detect a dominant clone in up to 2% of the samples in an extensive series of reactive T-cell samples, reflecting the good specificity of heteroduplex analysis (97.7%); the technique is particularly useful for distinguishing between erythrodermas of inflammatory origin and Sézary syndrome. The specificity of the BIOMED-2 protocols in the analysis of cutaneous samples of reactive origin or characteristics is high. Thus, faced with the need to confirm the presence of a T-cell clone in a reactive lymphoid infiltrate, study of the TCRβ gene, in view of its larger gene repertoire, may offer greater specificity.
Study of B-Lymphoid Clonality

Technical and Methodological Aspects

Study of rearrangement of the IgH gene is done using V_{H}, and J_{H} consensus primers for the framework regions (FR) I, FRII, or FRIII. The FRIII-J_{H} segment is the one most often studied for technical reasons; namely, the number of combinatorial possibilities is lower and the 2 segments are very close together in the rearranged gene (approximately 100 bp).

In an appropriate clinical-pathological context, in cases in which B-lymphoid clonality is not detected after PCR amplification of the FRIII-J_{H} segment, study of the FRII and FRI segments is recommended. However, the FRI segment is long and many more primers are needed for the study, making it often impossible to work with paraffin-embedded samples.15

The BIOMED-2 protocols include not just analysis of the complete VDJ gene of the IgH gene, but also the design of a PCR for studying incomplete DJ rearrangement.15 This segment is less subject to somatic hypermutations, which are common processes in mature B-cell malignancies.19 The high number of somatic hypermutations—which occur in V_{H} segments during transit of B cells through the germinal center—is responsible for a greater likelihood of primer hybridization errors and, therefore, of false negative results.

PCR methods have been described for amplification of the Igλ and Igκ genes, which in certain circumstances can yield greater sensitivity in the detection of B-cell clonality.62 Furthermore, κde segment rearrangement, which is also unaffected by somatic hypermutations, can be analyzed in addition to study of the complete Igκ gene. Rearrangement of κde is usually present in all Igκ malignancies and one-third of those of Igκ origin. Study of incomplete DJ rearrangement, as well as of rearrangement of the Igλ, Igκ, and κde genes, is used in those cases in which it has proved impossible to demonstrate the presence of a monoclonal B-cell population after analysis of the complete IgH (VDJ) gene (Figure 7).15

Results in Cutaneous B-Cell Lymphomas

Study of B-lymphoid clonality by PCR of the FRIII region can detect clonality in 30%-70% of B-cell lymphomas, depending on the type of sample and the laboratory technique used.1,27,63,64 Application of the BIOMED-2 protocol in sufficiently high-quality samples of systemic B-cell lymphomas, with simultaneous study of the FRI, FRII, and FRIII regions, managed to detect a clonal pattern in up to 90%-95% of cases. The presence of somatic hypermutations probably explains why clonality is not detected in all cases.15,16

The combination of study of complete IgH rearrangement (VDJ) with study of incomplete DJ rearrangements, as well as rearrangement of the light chains, has achieved almost 100% detection of clonality in cases of mantel-cell lymphomas, chronic lymphocytic leukemias, follicular lymphomas, and marginal zone lymphomas, and up to 98% detection in diffuse large B-cell lymphomas.15,16

For cutaneous B-cell lymphomas (CBCL), different studies have detected a sensitivity between 34% and 83% using PCR for B-cell clonality, generally with electrophoresis reading systems.42,63,65-70 It should be remembered that most of the studies published only analyze the FRIII region or segment, and so the rate of false negatives may be as high as 50%.64

Gellrich et al,71 in a recent study of paraffin-embedded samples of CBCL using BIOMED-2 protocols for amplification of the complete VDJ IgH gene (FRIII, FRII, FRI), found an overall clonality rate of 55.8% (100% for CBCL of the leg, 54.5% for marginal zone CBCL, and 66.7% for follicular CBCL).71 In that study, PCR for FRIII detected 46.5% clonality (100% for CBCL of the leg, 36.4% for marginal zone CBCL, and 40.7% for cutaneous follicular lymphomas). PCR of FRI only detected 20.9%, taking into account all samples (for 76% of the paraffin-embedded

Table 2. Criteria for Diagnosis of Incipient Mycosis Fungoides (International Society of Cutaneous Lymphoma)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Major (2 Points)</th>
<th>Minor (1 Point)</th>
</tr>
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<tbody>
<tr>
<td>Clinical</td>
<td>Persistent and/or progressive</td>
<td>2 y 1 y</td>
</tr>
<tr>
<td>Histologic</td>
<td>Macules or plaques + Unexposed area</td>
<td>Both Any</td>
</tr>
<tr>
<td>Molecular</td>
<td>Superficial lymphoid infiltrate + Epidermotropism without spongiosis Atypia</td>
<td>Present</td>
</tr>
<tr>
<td>Immunohistochemical</td>
<td>Monoclonal T-cell receptor rearrangement</td>
<td>CD2, CD3, CD5 &lt;50% CD7 &lt;10% Discontinuity between epidermis and dermis</td>
</tr>
</tbody>
</table>

Definitive diagnosis of mycosis fungoides is made for a score of 4 points or more.
samples, the level of quality control was below that recommended for amplification of the FRI fragment). However, if only amplifiable samples only were considered, PCR of FRI achieved a higher clonality detection rate (90%), which indicates that it is an excellent technique, particularly in fresh or frozen samples. Study of other FR regions seems to significantly increase sensitivity and would probably do so to a larger extent if incomplete DJ, Igλ, Igκ, and κδ rearrangements were also added to the B-cell genotypic analysis.15

Conclusion

The BIOMED-2 project for clonality analysis by GeneScan and heteroduplex analysis has proved to be a diagnostic strategy of high sensitivity and specificity for studying patients with cutaneous lymphoproliferative processes. Quality control and amplifiable DNA, particularly when obtained from material embedded in paraffin, is crucial for establishing whether a particular sample can be analyzed by a given PCR technique. The PCR study should be done in duplicate for each sample and, if possible, for 2 different samples from the same patient. If the clonality peak of the sample cannot be reproduced (with the same length), the result is not considered positive. This helps avoid the erroneous interpretation that a sample is monoclonal (false positives or pseudoclonality).

In the analysis of clonality of a lymphoid infiltrate of T cells, PCR amplification of TCRγ is studied initially. This gene, being a prototypical example of a short segment with few variable regions, is well preserved in archival paraffin-embedded samples. TCRγ is rearranged early in most T-cell malignancies, regardless of whether the phenotype is TCRαβ or TCRγδ. Given the greater complexity and laboriousness of the technique, study of rearrangement of the TCRβ gene represents a complementary strategy for those cases in which detection by TCRγ amplification has failed.

A strategic approach to sequencing in the study of B-cell clonality would be to start with study of the IgH gene in its FRIII segment first and subsequently study the FRII and FRI segments. Although the rates of detection of incomplete DJ, Igκ, Igλ, and κδ rearrangements are lower than in study of the complete VDJ gene, these are interesting complementary targets because they are unaffected by
hypermutations, which may be encountered particularly in marginal zone CBCL and follicular center CBCL.

Conflicts of Interest

The authors declare no conflicts of interest.

References


