Commentary

Advances in Molecular Hematopathology

*T-Cell Receptor γ and *bcl-2* Genes*

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The two articles by Signoretti et al¹ and Bohling et al² published in this issue of the *Journal* demonstrate ongoing advances in amplifying T-cell receptor γ gene rearrangements (TCR γ GR) and the translocation of the major breakpoint region of *bcl-2* with the immunoglobulin heavy chain gene (MBR/JH). Both use variations in polymerase chain reaction (PCR) technology. During the first few years' use of PCR in these two genes, analysis of PCR products occurred in standard agarose and polyacrylamide gels. There have been many technical advances since the 1988 description of manual PCR to detect MBR/JH rearrangements by Stetler-Stevenson et al.³ Recent progress has seen the utilization of fluorescent detection and methods of separating gene rearrangements focused on the differences in DNA sequences of the rearrangements. Three key methods are single-strand conformation polymorphism (SSCP) analysis,⁴ denaturing gradient gel electrophoresis (DGGE),⁵ and temperature gradient gel electrophoresis (TGGE).⁶ Pathologists interested in understanding the basics of DNA sequence separation technologies should read these three articles.

Principles of Analyzing T-cell Receptor γ Gene Rearrangements

One important feature facilitating the amplification of the T-cell receptor γ (TCR γ) gene is the limited number of variable region and joining region gene segments that have been described in tumors. These include four families of variable segments, V γ 1–8 (1–8 are closely related in sequence), V γ 9, V γ 10, and V γ 11, with three groups of joining segments, J γ 1–2, J γ P, and J γ P1–2.^{7–9} The total number of gene segments is much lower than in the T-cell receptor β or the immunoglobulin heavy chain genes and there are also no diversity gene segments.^{7–9} Therefore, it is easy for molecular pathologists to design primers to cover rearrangements of all of the variable region and joining region segments of the TCR γ gene. Each of these families or groups of segments requires a unique primer

because there is insufficient homology to use just one consensus primer for the variable region and one primer for the joining region genes.

A review of the literature reveals that there are two main groups of protocols: those which detail the amplification of all gene segments and those which amplify only the most common gene segments used in TCR γ GR. The most frequently used variable region and joining region segments in peripheral T cell lymphomas are the Group I variable region (V γ 1–8) at 80% and the J γ 1/J γ 2 group at 60%.⁹ Thus, common primers can detect approximately 60–80% of TCR γ GR according to the frequency of TCR gene segments used as described by in Theodorou et al.⁹ V γ 9, V γ 10, and V γ 11 are used in decreasing order, and J γ P and J γ P1/J γ P2 are used in less than 20% of TCR γ GR.⁹ The detection frequency of TCR γ GR with common primers in patient series tends to be higher than this due to the natural tendency to build a collection from positive index cases. It is also higher because biallelic rearrangements usually consist of one rearrangement composed of the commonly used segments and another composed of one of the infrequently used gene segments. Protocols using only a limited number of primers will fail to detect the TCR γ GR with the infrequently used genes. To test a protocol for detecting rearrangements with J γ P1/2, try amplifying DNA from MOLT4 cells.

In rearrangements of the immunoglobulin gene, maximum detection capabilities with Framework III primers are limited to about 80% due to a combination of mutations at primer sites and the presence of unknown gene segments. Unlike the immunoglobulin gene, we can expect to detect 90–100% of Southern blot-confirmed TCR γ GR when primers to all TCR γ gene segments are used.¹⁰ Therefore, I think pathologists are obligated to use all of the known TCR γ genetic information in designing protocols for their laboratory, regardless of whether SSCP, DGGE, TGGE, sequencing gels, heteroduplex, or high density polyacrylamide gels are used. To do less

Accepted for publication November 16, 1998.

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than that would, I believe, unnecessarily risk missing a TCR γ GR and may affect patient care.

The use of precast gels in thermally controlled SSCP (Signoretto et al¹ in this issue) to analyze T-cell receptor γ gene rearrangements is a good addition to recent descriptions of SSCP in various T-cell receptor gene families including α , β , and γ genes. Certainly the use of the precast gels, as also described by Kaul et al¹¹ using a PhastSystem (Pharmacia, Uppsala, Sweden), may be faster than pouring gels for either DGGE or SSCP systems. The goal of controlling temperature during gel electrophoresis is fundamentally important in any method for separating DNA by sequence to obtain high resolution of the DNA bands, as in SSCP. The use of SYBR Green II (Molecular Probes, Eugene, OR) appears to be a useful alternative to silver staining of single-stranded DNA in SSCP.

Alternative Gel Systems for TCR γ GR

Many methods have been described, ranging from early agarose gel-based electrophoresis, which separates DNA by length, to a variety of systems that separate DNA by sequences as well as length. A highly robust analytical electrophoresis system is desirable. The minimum standard is high density polyacrylamide gel electrophoresis to obtain sufficient discrimination between the amplicon products, which do not vary greatly in length with TCR γ due to the lack of a diversity region and limited insertions at the junction or N region. Electrophoresis assays based on DNA sequence separation are more robust in separating DNA, especially biallelic TCR γ GR of the same length.¹⁰ These are also useful in establishing with certainty that residual disease of a clonal neoplasm is present, based on a comigration in the gel of the rearranged band. The performance of sequence separation systems in mutation detection are nearly equivalent, yet remain dependent on specific DNA sequence and institutional expertise. Separation of TCR γ GRs in DGGE is maximized by the incorporation of a guanine-cytosine (GC) clamp in the PCR product.^{10,12} Representative reports suggest the use of TGGE in TCR γ GR¹³⁻¹⁵ appears equivalent to results seen in DGGE and SSCP. Newer methods involving fluorescent labeling of TCR β ¹⁶ and γ ^{17,18} GR PCR products with, eg, the ABI Gene Scan system (Applied Biosystems, Foster City, CA) a type of fluorescent SSCP, have been described; however, the instrumentation is very expensive. I and others have observed that this system discriminates very well by length¹⁶⁻¹⁸ and it has the added potential advantage of calculating comparisons between peak heights. This may prove useful in resolving a difficulty in all systems: discriminating oligoclonal expansions from neoplastic clones.

Fluorescent Detection System

The article by Bohling et al² describes a new way to detect PCR products that may lend itself to real time analyses, an elusive goal of several companies over the last five years. A recent advance in analyzing fluorescent-

labeled MBR/JH products included the application of the ABI Gene Scan (ABI, Molecular Probes) in a standard polyacrylamide gel.¹⁹ This system still represented a post-PCR electrophoresis. The fluorescent detection with SYBR Green I in the glass tube as described by Bohling et al,² however, uses properties of change in fluorescence that occurs with DNA strand association/disassociation coupled with measuring the temperature at which the DNA denatures. Such innovations are to be encouraged as laboratories continue changing from standard analyses of PCR products in labor-intensive agarose and polyacrylamide gels.

Background references that readers would find useful in understanding the technology described in this manuscript include the schematic article on the LightCycler instrument by Wittwer et al²⁰ and the principles of measuring DNA melting curves described by Ririe et al in 1997.²¹ These two articles break new ground in showing a potential application combining an understanding of DNA melting characteristics and fluorescent detection technology.

The application of this technology appears best suited for the detection of abnormal events such as neoplastic translocations, infectious disease agents, or mutations that have a significant difference in melting temperature. Members of Wittwer's group have previously illustrated a possible use in infectious diseases by amplifying the hepatitis B surface antigen²¹ with the LightCycler and others have demonstrated the capability in identifying *Leptospira* species.²² Most recently a LightCycler protocol has been published to identify a hemachromatosis gene mutation using yet another advance by incorporating the use of peptide nucleic acid probes²³ during PCR. Since amplified polyclonal JH or TCR γ rearrangements would affect fluorescent changes, it is not likely that this technique could be adapted easily in these areas.

The possibilities of incorporating this detection technology may be limited by the practice of confirming the identity of PCR products by hybridization techniques, such as dot blots or ELISA-based technology, along with the trend of directly sequencing PCR products. One concern about relying on DNA melting profiles to identify a specific product is that another DNA sequence may have the same melting temperature. A thorough understanding of DNA melting technology should, however, allow for the development of specific applications, such as the MBR/JH t(14;18) translocation, provided there are no known nonspecific products such as cross-reaction with Epstein-Barr virus.²⁴

One slight drawback with previous generations of rapid cyclers, which we have experienced in our laboratory, is difficulty in loading and handling the capillary tubes. The innovators have attempted to address this with a new plastic loading unit for the capillary tubes that may make it more user friendly.²

The Problem of Sporadic bcl-2 MBR/JH Rearrangements

MBR/JH rearrangements have been described in individuals with no disease or with reactive hyperplasia.^{25,26} The

best way to identify sporadic bcl-2 translocations is by duplicate tube analysis and they can usually be avoided by using a nonnested protocol.²⁷ If the suspect translocation is not repeatable in multiple tube assays it is best regarded as sporadic when one is considering a primary diagnosis. In follow-up biopsy specimens in an established patient the best course of action is to use comparative analysis for comigration of MBR/JH in the two biopsy specimens.

Future of Molecular Hematopathology

Advances during the past decade have resulted in numerous protocols for detecting gene rearrangements and translocations in lymphoma and leukemia by PCR. Therefore, gel systems and primers vary from institution to institution, making comparisons difficult. The Association for Molecular Pathology and the National Committee on Clinical Laboratory Standards are making ongoing efforts to determine whether consensus protocols can be devised.²⁸

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