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T-cell non-Hodgkin lymphomas are uncommon, representing approximately 10 percent of all non-Hodgkin lymphomas (1). For pathologists, the diagnosis of T-cell lymphomas is frequently challenging, with a differential diagnosis that includes benign reactive lymphoid proliferations. Documentation of a clonal T-cell population is therefore often an important part of the workup of atypical T-cell-rich lymphoid proliferations.

T-cells recognize antigen in the context of the major histocompatibility complex (MHC) molecules through the T-cell receptor (TCR) (2). Two major forms of TCR may be found in normal T-cells, each of which has a heterodimeric structure. Approximately 90 percent of T-cells express an alpha-beta TCR, composed of one alpha and one beta chain. The remaining 10 percent of T-cells express a gamma-delta TCR, composed of one gamma and one delta chain. The TCR alpha and delta genes are organized in one combined locus (TCRAD) on chromosome 14q11, while the TCR beta (TCRB) and gamma (TCRG) chains are located on chromosomes 7q34 and 7p14, respectively. The structure of these loci, similar to those for the immunoglobulin molecules expressed by B-cells, is complex.

**Diverse Receptor Population**

To be able to respond to as many foreign antigens as possible, each T-cell precursor develops an essentially unique TCR molecule. This remarkable diversity of TCR proteins is due in part to a phenomenon known as combinatorial diversity. Instead of a typical contiguous exon/intron genetic structure, TCR loci contain numerous gene segments that undergo recombination during T-cell development to produce a final, rearranged gene (2).

Specifically, the TCRB locus contains about 65 V (variable) segments, two D (diversity) segments, and 13 J (joining) segments in addition to two constant-region gene segments. During recombination, one V segment, one D segment, and one J segment are selected and placed in proximity to each other upstream of the constant region while the intervening DNA is deleted. This process produces a final recombinated VDJ-constant region TCRB gene.

Similarly, the combined TCRAD locus contains approximately 75 Va segments, 10 Vδ segments, 60 Jα segments, two Jδ segments, two Dδ segments, and one constant-region gene segment each for alpha and delta. The TCRG locus is the simplest of all of the T-cell loci, with 11 V segments, two J segments, and two constant-region segments. Furthermore, during the recombination process, additional random nucleotides are frequently inserted at the junctional breakpoints, a phenomenon known as “junctional diversity.” Considering all the possible combinations of gene segments and frequency of junctional diversity, the total potential T-cell receptor repertoire is approximately $10^{16}$ TCR alpha-beta complexes and $10^{18}$ TCR delta-gamma complexes.

During a benign, reactive T-cell response, multiple T-cells, each with their own rearranged TCR loci, are proliferating. In contrast, in a T-cell lymphoma, the original neoplastic cell gives rise to a population of cells that all display an identical TCR rearrangement.

**Early Molecular Approaches**

Molecular methods to determine whether a T-cell proliferation is polyclonal or monoclonal began in the 1980’s with Southern blot analysis. In this technique, DNA is digested by restriction enzymes and probed with DNA complementary to the TCR locus of interest, typically the TCRB constant region. In a polyclonal proliferation, the rearranged loci are of variable lengths, producing a smear on the Southern blot. In a
monoclonal population, the rearranged cells all display fragment lengths of uniform size, leading to a discrete band. Southern blot analysis theoretically is capable of detecting all monoclonal T-cell rearrangements, but several factors limit the applicability of this technique. First, the technique is time-consuming and labor-intensive, typically requiring four to seven working days. Secondly, high molecular weight DNA is required, which can be produced only from fresh or frozen tissue, not from formalin-fixed material. Southern blot studies, therefore, cannot be used in many cases where only formalin-fixed, paraffin-embedded (FFPE) tissue is available.

The PCR Advantage

PCR-based techniques for T-cell receptor rearrangements arose in the 1990’s. Most labs chose to employ consensus primers for the TCRG locus, which was chosen specifically for two reasons. First, the relatively simple organization of this locus (only 11 V segments, compared to >50 TCRAD V segments) was well-suited to primer design. Second, during the process of T-cell development, the TCRG locus is the first to undergo rearrangement. T-cells that fail to successfully rearrange the TCRG locus may go on to rearrange the TCRB locus. For these reasons, essentially all mature T-cells have a rearranged TCRG locus, regard-

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**PCR for T-cell clonality assessment using BIOMED-2 primers**

Examples of polyclonal and monoclonal T-cell populations are shown using three multiplexed primer sets targeting the TCRB locus (A and B, Vβ-Jβ rearrangements; C, Dβ-Jβ rearrangements). In polyclonal populations, multiple peaks are detected with each primer set. In monoclonal populations, a single, dominant peak is identified.
less of whether the cell expresses TCR alpha-beta or TCR gamma-delta proteins. Using primers for the Vγ and Jγ regions, one can analyze the diversity of rearrangements present. In a polyclonal population, many different-sized VDJ rearrangements were observed, while a monoclonal population gave rise to a single peak (Figure 1).

Importantly, such PCR studies amplify smaller DNA fragments that can be obtained out of FFPE material. In practice, the TCRG primers employed by most labs until recently were capable of detecting monoclonal rearrangements in approximately 90 percent of T-cell lymphomas; in the remaining cases, Southern blot studies remained necessary for clonality determination.

**Improving on PCR: BIOMED-2**

To improve the sensitivity of PCR-based clonality detection, a consortium of European molecular diagnostic laboratories developed the BIOMED-2 series of multiplexed primers to assess clonality at the TCRB and TCRG loci with sensitivity equal to Southern blot studies (3, 4). In conjunction with the Cleveland Clinic Pathology & Laboratory Medicine Institute Center for Test Development, our Department of Molecular Pathology now has adopted the BIOMED-2 primer sets for routine analysis.

This new assay employs three multiplexed reactions using primers for the TCRB locus (two tubes for Vβ-Jβ rearrangements, one for Dβ-Jβ rearrangements) and two multiplexed reactions for primers covering the TCRG locus (each targeting specific Vγ-Jγ rearrangements). This combination of multiplexed primer sets has been demonstrated to detect >98 percent of TCR rearrangements, significantly improving the utility of PCR-based clonality assessment. These studies may greatly assist in the diagnosis of T-cell lymphomas and will decrease the need for second surgeries, which previously may have been required to obtain fresh tissue for Southern blot analysis.

**Limitations of the Assay**

While the BIOMED-2 approach has decreased the false negative rate of PCR-based clonality studies, pathologists must be aware of the limitations of any technique for clonality assessment (5). Most importantly, clonality is not always equivalent to malignancy: occasional reactive T-cell proliferations may show an oligoclonal or even monoclonal proliferation. In addition, other neoplasms, including B-cell lymphomas or myeloid leukemias, occasionally may show clonal rearrangements of the immunoglobulin loci. Therefore, interpreting BIOMED-2 PCR studies in the context of other histologic and phenotypic studies remains very important for final evaluation. With such a multiparameter approach, patients with possible T-cell lymphomas will achieve the best possible diagnosis, allowing for appropriate management.

**References**


**Collaborative New Test Development is a Win-Win**

Validation of the BIOMED-2 T-cell clonality assay is one of the first collaborative projects between a clinical section in Pathology & Laboratory Medicine Institute (PLMI) and the Center for Test Development (CTD). James Cook, MD, PhD, and Gurunathan Murugesan, PhD, worked closely to develop an initial validation plan for the BIOMED-2 assay, incorporating the appropriate studies to meet the clinical need. Research technologist Kristen McDonnell performed experiments in the CTD laboratory to generate the data necessary to validate the analytical performance of the assay. She then provided a final validation report and draft SOP for the clinical laboratory.

This project is an example of how the CTD can serve as a dedicated resource for personnel, equipment and space to support test development across all sections of PLMI.

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BK viral nephropathy is a potential complication of the immunosuppression required for successful renal transplantation. The nephropathy is thought to arise via reactivation of latent BK virus resulting from sustained immunosuppression. Graft loss will occur if the nephropathy is not managed appropriately. Recognition of BK viral nephropathy is essential to the successful management of immunosuppression in patients undergoing renal transplantation, since the appropriate treatment for BK viral nephropathy is usually a reduction in immunosuppression. Conversely, if the underlying cause of increasing renal insufficiency is acute allograft rejection, the appropriate response is usually to increase immunosuppression.

In most renal transplant patients, urine usually is qualitative to determine if the virus is present, and plasma is quantitative to monitor viral load. BK quantitative viral load plasma monitoring (or qualitative urine monitoring) by real-time polymerase chain reaction provides sufficient laboratory data for effective management. Renal transplant biopsies are performed either as part of a standard transplant protocol or to clarify the underlying cause of increasing renal insufficiency.

Tubular epithelial intranuclear viral inclusions can be recognized in many, if not most, cases by conventional histopathologic examination (Figure 1). Four different variants of intranuclear inclusions associated with BK nephropathy have been described, including the most common amorphous, basophilic, ground-glass variant; an eosinophilic, granular halo-encircled inclusion; a finely granular type lacking a halo; and a vesicular variant with markedly enlarged nuclei and clumped, irregular chromatin. The distal nephron tubular epithelium is most commonly involved in BK infection. BK-infected cells may become rounded-up and extruded from the epithelial cell layer into the tubular lumen.

In situ hybridization is a valuable ancillary tool to confirm the presence or absence of BK viral nephropathy in renal transplant biopsy specimens. Fluorescence in situ hybridization (FISH) for BK virus can be used to confirm the presence of the virus in renal tubular epithelial cell nuclei (Figure 2).

The BK virus is one of three polyoma viruses (BK, JC, and Merkel) in the family polyomaviridae. The human BK virus is a relatively small, common non-enveloped, double-stranded DNA virus. Lytic destruction of infected tubular renal epithelium induces an influx of activated T lymphocytes similar to the interstitial infiltrate of T-cells associated with acute allograft rejection.

The Cleveland Clinic Department of Molecular Pathology has a FISH assay for detection of BK viral DNA sequences in infected tissue. The assay format utilizes an external positive control to determine the sensitivity of the assay and to confirm the absence of background hybridization signal in non-infected tissue. The assay is performed on formalin-fixed, paraffin-embedded tissue sections using a specific probe for BK viral DNA.

**Figure 1:** Photomicrograph, renal allograft biopsy, demonstrating nuclear inclusions in tubular epithelial cell nuclei consistent with BK virus cytopathic effect. Hematoxylin and eosin, x 400.
control (renal allograft tissue with previously established BK viral nephropathy) that is probed using 1) BK-specific sequences, and 2) the pBR322 negative control probe (plasmid DNA without the specific BK complementary DNA sequences). The pBR322-BKV-Dunlop plasmid containing the BK virus T-ag and t-ag was generously provided by Janet S. Butel, PhD, Department of Molecular Virology and Microbiology, Baylor College of Medicine (Figure 3).

The plasmid was transformed to the E.coli DH5-alpha and incubated in LB medium containing ampicillin following the laboratory protocol. The plasmid then was extracted using the Qiagen QIAfilter Plasmid Midi Kit (Qiagen, Valencia, Calif.) and labeled using the Vysis Nick Translation Kit (Abbott Molecular, Des Plaines, Ill.) according to the manufacturer’s protocol. Specifically, a 50 ul mixture containing 1 ug plasmid DNA, 2.5 ul 0.2mM SpectrumRed, 5 ul of 0.1mM dTTP, 10 ul of 0.1mM dNTP mix, 5 ul 10X nick translation buffer, and 5 ul nick translation enzyme was incubated on a thermal cycler at 15°C for eight hours. The pBR322-BKV-Dunlop plasmid was co-labeled together with its vehicle as a control. The probes generated were purified using the Ambion NucAway™ Spin Columns (Applied Biosystems/Ambion, Austin, Texas) and precipitated using 2.5 volumes of 100% EtOH and 0.1 volume 3 M sodium acetate overnight. The pellet was resuspended in 10 ul of Sigma molecular-grade water and stored at -20°C covered in foil.

The renal biopsy specimen is probed with BK-specific DNA labeled with SpectrumRed that produces very bright red fluorescence upon excitation. The probe cocktail also contains a SpectrumGreen-labeled CEP8 centromeric probe as an internal control for successful hybridization. A separate negative control slide is probed with the pBR322 plasmid control that does not have BK complementary sequences; this negative control probe has been labeled with SpectrumRed and also contains CeP8-SpectrumGreen as a control for successful hybridization. BK viral sequences are detected as intense red fluorescence within tubular epithelial nuclei (Figure 2); one or two green centromeric signals for chromosome 8 confirm successful hybridization within both infected and noninfected nuclei.

Results are reported qualitatively as FISH for BK virus “positive” or “negative.” The FISH assay is intended to be used as an ancillary test interpreted in the context of conventional morphological evaluation of the renal biopsy.

References
USCAP 2010 in Review

The Department of Anatomic Pathology continues to be a dominant presence in the US & Canadian Academy of Pathology (USCAP). Department faculty and fellows presented a total of 65 poster and platform sessions at the 2010 Annual Meeting, and faculty monitored two of the platform sessions. Faculty also were invited to present seven short courses, two companion society courses and two evening conferences.

Second-year resident Manju Aron, MD, won the ADASP/USCAP Autopsy Award for Pathologists-in-Training for the poster “Acute aortic dissection: changing spectrum of clinicopathologic findings at autopsy.” In addition to Dr. Aron, the poster was authored by E. Rene Rodriguez, MD, and Carmela Tan, MD.

As part of the Department of Anatomic Pathology’s commitment to research and education, department staff members are very active in the organization. Department Chair John Goldblum, MD, chairs the USCAP education Committee and serves as the course director for the USCAP Practical Pathology Seminars course. Mary Bronner, MD, is a member of the USCAP Council. James Cook, MD, PhD, Cristina Magi-Galluzzi, MD, Lisa Yerian, MD, and Dr. Rodriguez are on the USCAP Abstract Review Board.

New Real-Time PCR Assay for BCR/ABL Now Available

The Department of Molecular Pathology now offers a new, qualitative RT-PCR assay for the p190 (e1a2) isofrom of the BCR/ABL translocation associated with Philadelphia (Ph) chromosome-positive acute lymphoblastic leukemia (ALL). Three assays are now available for the detection of BCR/ABL.

FISH for BCR/ABL (BCRFSH) - detects the p210 (e13a2/e14a2), p190 (e1a2) or other rare variant isoforms of BCR/ABL. Peripheral blood FISH for BCR/ABL is recommended for screening for chronic myeloid leukemia (CML). FISH is not recommended for detection of post-treatment minimal residual disease.

BCR/ABL p210 RT-PCR, quantitative (BCRPCR) - for routine monitoring of patients with CML on tyrosine kinase inhibitor therapy. Analysis of a pre-treatment baseline sample is also suggested, when possible. Results are reported as the percentage ratio of BCR/ABL fusion transcripts to wild type ABL transcripts (e.g. a ratio of 0.062 is now reported as 6.2%). Results also will continue to be reported as log reduction from baseline.

BCR/ABL p190 RT-PCR, qualitative (190BCR) - for detection of minimal residual disease in Ph chromosome-positive ALL (sensitivity of 10-5). A pre-treatment baseline is also suggested, when possible, to document the presence of the p190 transcript.

Direct technical questions regarding these assays to Kelly Lyon, 216.444.8283 or palinck@ccf.org. For questions regarding clinical indications for or interpretation of these assays, please contact James Cook, MD, at 216.444.4435 or cookj@ccf.org or Eric Hsi, MD, at 216.444.5230.

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