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Evaluation of Positive B- and T-Cell Gene Rearrangement Studies in Patients With Negative Morphology, Flow Cytometry, and Immunohistochemistry

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The diagnosis of a lymphoid malignancy requires the establishment of monoclonality of a lymphocyte population through morphologic assessment and laboratory testing, such as flow cytometry, immunohistochemistry, and cytogenetic analysis. These methods can yield conflicting results, however, and in up to 15% of cases of suspected lymphoid malignancies, molecular assessment of immunoglobulin (IG) and/or T-cell receptor (TCR) gene rearrangements is necessary to confirm a diagnosis. IG/TCR gene rearrangement testing is based on the principle due to V(D)J recombination in lymphocytes, the probability a population of B or T cells would share the same IG or TCR gene rearrangement is virtually zero. As such, the presence of a population of B or T cells with the same gene rearrangement pattern is highly supportive of lymphoid malignancy.

Modern IG/TCR gene rearrangement analysis generally consists of manual or automated extraction of target DNA (eg, from peripheral blood, bone marrow aspirate, fresh or frozen tissue, or formalin-fixed, paraffin-embedded tissue) followed by multiplex polymerase chain reaction (PCR) and capillary gel electrophoresis. Standardized primer sets and protocols are commercially available, as are guidelines for a standardized approach to interpreting and reporting gene rearrangement results. When IG/TCR clonality assessment is performed using these standardized reagents and protocols, detection of nearly all lymphoid malignancies is possible, including 99% of B-cell clones and 94% of T-cell clones.

At times, results from morphologic assessment and other ancillary testing are benign or unrevealing despite the presence of an (apparent false) positive IG or TCR gene rearrangement result. The significance of positive rearrangement studies in the context of otherwise normal morphology and flow cytometry is unknown, and as such, we hypothesized gene rearrangement studies may be predictive of an emerging B- or T-cell clone in the absence of other abnormal laboratory tests, given the potentially higher sensitivity of molecular methods to detected abnormal...
platforms. Categorized as test positive/phenotype positive (T+), cytogenetic characterization of the tissue source were recorded from the electronic medical record. Date of testing, specimen Hospital between January 1, 2013 and July 6, 2018 were extracted (patients white (82%), 19 African American (14%), 2 Native American (98%) and 3 women (2%). Self-reported race was 111 characteristics are found in Table 1. The patients were 133 studies (50 PB and 153 BM), were analyzed. Demographic were 2 T need for involvement of prior Waldenstrom’s macroglobulinemia. Of these 3 patients, 1 later developed acute myeloid leukemia. None of them subsequently developed a lymphoproliferative disorder and the patient with Waldenstrom’s was not noted to develop relapse of his disease during the study period. The nine T+ results in 99 BM studies were identified, again using criteria established in methods. Sensitivity and specificity, respectively, were calculated for PB TCR studies (94% and 93%), BM IG studies (71% and 95%), and BM TCR studies (92% and 83%). Analysis of PB IG gene rearrangement studies was not performed because of the small number of tests (3; all T−). Characteristics of 12 cases associated with T+/P− IG and TCR gene rearrangement studies are found in Table 3. The 3 T+−/P− IG studies corresponded to nonmalignant diagnoses, including 1 normal marrow, 1 indeterminate result, and 1 result negative for involvement of prior Waldenstrom’s macroglobulinemia. Of these 3 patients, 1 later developed acute myeloid leukemia. None of them subsequently developed a lymphoproliferative disorder and the patient with Waldenstrom’s was not noted to develop relapse of his disease during the study period. The nine T+−/P− TCR results corresponded to 4 normal/nonmalignant pathology reports, 1 negative result for BM involvement of clear cell carcinoma of the kidney, 1 diagnosis of aplastic anemia, 1 diagnosis of polycythemia vera, 1 diagnosis of lymphohistiocytosis, and 1 indeterminate result. Of these patients, 1 subsequently developed acute myeloid leukemia, and none of the patients subsequently developed a lymphoproliferative disorder. For the 12 cases with T+/P− results, the time of follow-up surveillance after the first positive rearrangement study ranged from 23 to 1284 days, with a mean of 363 days and median of 210 days between the initial positive study and last opportunity for surveillance for emergence of a lymphoproliferative disorder. One patient who died from hemophagocytic lymphohistiocytosis shortly after an initial T+/P− gene rearrangement analysis was excluded from this calculation.

### RESULTS

A total of 136 patients, who had 203 gene rearrangement studies (50 PB and 153 BM), were analyzed. Demographic characteristics are found in Table 1. The patients were 133 men (98%) and 3 women (2%). Self-reported race was 111 patients white (82%), 19 African American (14%), 2 Native Hawaiian/Pacific Islander (1%), and 1 Native American (<1%), with 3 patients declining to answer (2%). Self-reported ethnicity was 116 non-Hispanic (85%) and 5 Hispanic (4%), with 15 patients declining to answer (11%). The mean age at time of first testing was 69 years. Results from IG and TCR gene rearrangement studies from PB and BM are found in Table 2. In TCR studies, there were 2 T+/P− and 1 T−/P− results in 47 PB assays using criteria established in methods, as well as 7 T+/P− and 1 T−/P+ results in 54 BM assays. Regarding IG studies, 3 T+/P− and 12 T−/P+ results in 99 BM studies were identified, again using criteria established in methods. Sensitivity and specificity, respectively, were calculated for PB TCR studies (94% and 93%), BM IG studies (71% and 95%), and BM TCR studies (92% and 83%). Analysis of PB IG gene rearrangement studies was not performed because of the small number of tests (3; all T−/P−).

### METHODS

Data from all patients who underwent IG or TCR gene rearrangement testing at the authors’ affiliated Veterans Affairs Hospital between January 1, 2013 and July 6, 2018 were extracted from the electronic medical record. Date of testing, specimen source, and morphologic, flow cytometric, immunohistochemical, and cytogenetic characterization of the tissue source were recorded from pathology reports. Positive gene rearrangement results were categorized as test positive/phenotype positive (T+/P+) if they corresponded with a pathologic diagnosis of a lymphoproliferative disorder, and test positive/phenotype negative (T+/P−) if they corresponded with normal or nonmalignant pathology results. Negative gene rearrangement results were categorized as test negative/phenotype negative (T−/P−) if they corresponded to nonmalignant diagnoses, or test negative/phenotype positive (T−/P+) if they corresponded to a pathologic diagnosis of a lymphoproliferative disorder. Last, patient records were reviewed for subsequent diagnosis of hematologic malignancy in patients with positive gene rearrangement results with negative ancillary testing. All gene rearrangement studies were performed according to BIOMED-2 protocol using fresh samples from peripheral blood (PB) and bone marrow (BM).

### Table 1. Demographics of the Study Population

| Characteristic            | Total Number | Percentage |
|---------------------------|--------------|------------|
| Total number of patients  | 136          |            |
| Mean age at first testing | 69.13        |            |
| Sex                       |              |            |
| Male                      | 133          | (98%)      |
| Female                    | 3            | (2%)       |
| Race                      |              |            |
| White                     | 111          | (82%)      |
| African American          | 19           | (14%)      |
| Native American           | 1 (<1%)      |            |
| Native Hawaiian/Pacific Islander | 2 (1%) |            |
| Declined to answer/data absent | 3 (2%) |            |
| Ethnicity                 |              |            |
| Hispanic                  | 5 (4%)       |            |
| Non-Hispanic              | 116 (85%)    |            |
| Declined to answer/data absent | 15 (11%) |            |
| Total number of gene rearrangement tests | 203 |            |
| Number of tests from peripheral blood | 50 |            |
| Number of tests from bone marrow | 153 |            |

### Table 2. Immunoglobulin and T-Cell Receptor Gene Rearrangement Results From Peripheral Blood and Bone Marrow

| Group          | PB IG | PB TCR | BM IG | BM TCR |
|----------------|-------|--------|-------|--------|
| T+/P+          | 0     | 17     | 30    | 12     |
| T+/P−          | 0     | 2      | 3     | 7      |
| Total Positive Tests | 0   | 19     | 33    | 19     |
| T−/P+          | 0     | 1      | 12    | 1      |
| T−/P−          | 3     | 27     | 54    | 34     |
| Total Negative Tests | 3   | 28     | 66    | 35     |
| Total malignant | 0     | 18     | 42    | 13     |
| Total nonmalignant | 3     | 29     | 57    | 41     |
| Total Tests    | 3     | 47     | 99    | 54     |

Abbreviations: BM, bone marrow; IG, immunoglobulin; PB, peripheral blood; T+/P+, test positive/phenotype positive; T+/P−, test positive/phenotype negative; T−/P+, test negative/phenotype negative; T−/P−, test negative/phenotype positive; TCR, T-cell receptor.
ed to 1 diagnosis of T-cell acute lymphoblastic leukemia and 1 diagnosis of T-cell large granular lymphocytic leukemia.

**DISCUSSION**

Results from this study support the general recommendation that PCR-based IG and TCR gene rearrangement analysis should be interpreted in the context of clinical, morphologic, immunohistochemical, and cytogenetic data in order to establish the diagnosis of a lymphoproliferative disorder. Contrary to our hypothesis, none of the patients with an isolated positive IG or TCR gene rearrangement result subsequently developed a lymphoproliferative disorder, suggesting these gene rearrangement results were not predictive of a burgeoning malignancy undetected by other tests.

Clonal lymphocyte populations can be detected in nonmalignant settings, leading to T+/P− gene rearrangement results. For example, monoclonal and oligoclonal T-cell populations can be detected by PCR in viral infections, benign skin disorders, advanced age, and recovery from chemotherapy or hematopoietic stem cell transplantation. Similarly, clonal B-cells can be observed in immunosuppression or autoimmune disease. In the setting of minimal residual disease monitoring, a monoclonal B-cell population may develop additional IGH gene rearrangements through the process of clonal evolution, resulting in T+/P− (ie, a new gene rearrangement is detected that appears to be a new monoclonal population but merely represents a new arrangement in the same monoclonal population) or T−/P+ (ie, the presence of new gene rearrangements obscures the original rearrangement associated with the monoclonal population) PCR results. Notably, of the 14 T−/P+ gene rearrangement results observed in this study, 10 (71%) were related to a diagnosis of plasma cell neoplasms. Because plasma cells are B cells, IG gene rearrangement analysis can be used to establish clonality in plasma cell neoplasms, such as MM. In rare subtypes of MM, such as nonproducer, nonsecretor MM, IG gene rearrangement analysis may at times be the only laboratory assay capable of demonstrating plasma cell monoclonality. Somatic hypermutation of IGH genes in postgerminatal center B-cell malignancies, including MM is a well-established cause of T−/P+ gene rearrangement analysis, requiring IGK rearrangement analysis to reliably establish clonality in the setting of these diseases. In all 10 T−/P+ cases related to plasma cell neoplasms in the present study, gene rearrangement analysis was performed on IGH only. The addition of IGK analysis in these cases likely would have decrease the rate of observed T−/P+ results.

Other technical and biological limitations underlie T−/P+ gene rearrangement results. For example, PCR-based gene rearrangement analysis has been shown in some studies to fail to detect monoclonal lymphocyte populations with less than 5% to 10% of clonal cells, and as such, T−/P+ results can be found in the presence of small malignant lymphocyte populations. Occasional T−/P+ also result from the exclusion of select complex gene rearrangements in BIOMED-2 primer sets in order to avoid an unacceptable rate of T+/P− results. Similarly, PCR products falling outside of the 5th or 95th percentiles of size can rarely represent true monoclonal lymphocyte populations despite being regularly excluded from analysis. Last, chromosomal

### Table 3. Characteristics of Test Positive/Phenotype Negative Gene Rearrangement Results

| IG or TCR Testing | Specimen Source | Diagnosis Resulting From Testing | Time to Most Recent Follow-Up | Subsequent Diagnosis |
|-------------------|-----------------|---------------------------------|-----------------------------|---------------------|
| IG                | BM              | Normal/nonmalignant             | 650 d                       | —                   |
| IG                | BM              | Indeterminate                   | 1284 d                      | AML                 |
| IG                | BM              | No bone marrow involvement of WM of lymph node | 364 d | — |
| TCR               | BM              | Nonmalignant: HLH               | 76 d                        | —                   |
| TCR               | BM              | Normal/nonmalignant             | 23 d                        | —                   |
| TCR               | BM              | Myeloproliferative neoplasm: PV | 157 d                      | AML                 |
| TCR               | BM              | Nonmalignant: aplastic anemia   | 28 d                        | —                   |
| TCR               | BM              | Normal/nonmalignant             | 251 d                       | —                   |
| TCR               | PB              | Normal/nonmalignant             | 152 d                       | —                   |
| TCR               | PB              | Indeterminate                   | 210 d                       | —                   |

### Table 4. Characteristics of Test Negative/Phenotype Positive Gene Rearrangement Results

| IG or TCR Negative | Specimen Source | Diagnosis Resulting From Testing |
|--------------------|-----------------|---------------------------------|
| IG                 | BM              | MM                              |
| IG                 | BM              | MM                              |
| IG                 | BM              | MM + low-grade B NHL NOS        |
| IG                 | BM              | MGUS                            |
| IG                 | BM              | MM                              |
| IG                 | BM              | MBL                             |
| IG                 | BM              | Low-grade B NHL NOS             |
| IG                 | BM              | MM                              |
| IG                 | BM              | Low-grade B NHL NOS (+ MDS)     |
| IG                 | BM              | MGUS                            |
| IG                 | BM              | MGUS (+ ATLL)                   |
| TCR                | BM              | T-ALL                           |
| TCR                | PB              | T-LGLL                          |

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; HLH, hemophagocytic lymphohistiocytosis; IG, immunoglobulin; PB, peripheral blood; PV, polycythemia vera; TCR, T-cell receptor; WM, Waldenstrom’s macroglobulinemia.
abnormalities, such as t(11;14) and t(14;18), in B-cell neoplasms are not amplified in IGH multiplex PCR and can also cause T-/P+ IG gene rearrangement studies.

This study is subject to important limitations. First, these data were obtained from the electronic medical record of a single clinical site with a primarily male patient population and may not reflect the clinical or laboratory practices of other sites. Additionally, it is possible some patients may have developed a subsequent lymphoproliferative disorder and presented to another hospital than the one examined in this study. Nonetheless, as a chronic care center, we believe our Veterans Affairs facility helps to limit this possibility, as patients seen often maintain some aspect of their care within our system, which would have led to at least updating of their medical problem list (if not frank treatment at our hospital). Last, living patients with positive gene rearrangement results may still develop a lymphoproliferative disorder the time constraints of the study did not allow to be detected, and it may be warranted for us to re-examine the T+/P– patient cohort in 5 years to see if there is any new evidence for development of lymphoproliferative processes.

In summary, the results from the present study suggest positive IG/TCR gene rearrangement studies are not predictive of lymphoproliferative disorders in the context of otherwise negative BM or PB findings. As such, when faced with equivocal pathology reports, clinicians can be practically advised that isolated positive IG/TCR gene rearrangement studies do not indicate a need for closer surveillance.

References
1. Woo J, Baumann A, Arguello V. Recent advancements of flow cytometry: new applications in hematology and oncology. Expert Rev Mol Diagn. 2014;14(1):67–81.
2. van Krieken JH, Langerak AW, Macintyre EA, et al. Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 concerted action BMH4-CT98-3936. Leukemia. 2007;21(2):201–206.
3. Fan H, Rubertoye RS. Detection of clonal immunoglobulin heavy chain gene rearrangements by the polymerase chain reaction and capillary gel electrophoresis. In: Cazader M, ed. Hematological Malignancies: Methods and Protocols. New York: Humana Press; 2013:151–167.
4. Fan H, Rubertoye RS. Detection of clonal T-cell receptor beta and gamma chain gene rearrangement by polymerase chain reaction and capillary gel electrophoresis. In: Cazader M, ed. Hematological Malignancies: Methods and Protocols. New York: Humana Press; 2013:169–188.
5. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspected lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17(12):2257–2317.
6. Langerak AW, Groenen PJ, Bruggemann M, et al. EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. Leukemia. 2012;26(10):2159–2171.
7. Langerak AW, Molina TJ, Lavender FL, et al. Polymerase chain reaction-based clonality testing in tissue samples with reactive lymphoproliferations: usefullness and pitfalls. A report of the BIOMED-2 concerted action BMH4-CT98-3936. Leukemia. 2007;21(2):222–229.
8. Evans PA, Pott C, Groenen PJ, et al. Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets. Report of the BIOMED-2 concerted action BMH4-CT98-3936. Leukemia. 2007;21(2):210–214.
9. Hodges E, Krishna MT, Pickard C, Smith JL. Diagnostic role of tests for T cell receptor (TCR) genes. J Clin Pathol. 2003;56(1):1–11.
10. Bekkenk MW, Geelen FA, van Voorst Vader PC, et al. Primary and secondary cutaneous CD30(+)/CD45(-) lymphoproliferative disorders: a report from the Dutch Cutaneous Lymphoma Group on the long-term follow-up data of 219 patients and guidelines for diagnosis and treatment. Blood. 2000;95(12):3653–3661.
11. Sorzotti M, Patel DD, Li X, et al. T cell repertoire development in humans with SCID after nonablative allogeneic marrow transplantation. J Immunol. 2003;170(5):2711–2718.
12. Mariani S, Coscia M, Even J, et al. Severe and long-lasting disruption of T-cell receptor diversity in human myeloma after high-dose chemotherapy and autologous peripheral blood progenitor cell infusion. Br J Haematol. 2001;113(4):1051–1059.
13. Lee SC, Berg KD, Racke FK, Griffin CA, Eshleman JR. Pseudo-spikes are common in histologically benign lymphoid tissues. J Mol Diagn. 2000;2(3):145–152.
14. Dunlap JB, Fan G, Leeborg N, Braziel RM. B-Cell Malignancies. In: Leonard DGB, ed. Molecular Pathology in Clinical Practice. 2nd ed. Switzerland: Springer International Publishing; 2016:579–602.
15. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 2014;15(12):e538–e548.
16. Mendoza H, Torrey CA, Siddon AJ. Use of B-cell gene rearrangement studies to establish clonality in non-producer non-secretory multiple myeloma. Clin Lymphoma Myeloma Leuk. 2010;20(11):e138–e21.
17. Payne K, Wright P, Grant JW, et al. BIOMED-2 PCR assays for IGK gene rearrangements are essential for B-cell clonality analysis in follicular lymphoma. Br J Haematol. 2011;151(1):84–92.
18. Kokovic I, Jezerski Novakovic B, Novakovic S. Diagnostic value of immunoglobulin kappa light chain gene rearrangement analysis in B-cell lymphomas. Int J Oncol. 2015;46(3):953–962.
19. Langerak AW. Undersized, oversized? It is not one-size-fits-all in lymphoid clonality detection. Leuk Res. 2008;32(2):203–204.