PCR Assays Detect B-Lymphocyte Clonality in Formalin-Fixed, Paraffin-Embedded Specimens of Classical Hodgkin Lymphoma Without Microdissection

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Key Words: Hodgkin lymphoma; Immunoglobulin genes; Polymerase chain reaction; PCR; Clonality

Abstract

Hodgkin lymphoma (HL) was shown to be a B-cell malignancy using polymerase chain reaction (PCR) clonality studies of microdissected Reed-Sternberg cells. While methods for the detection of B-cell clonality could aid in the diagnosis of HL, microdissection is not practical in most clinical settings. We assessed the standardized BIOMED-2 IGH and IGK PCR primers for the detection of clonality using 50 consecutively diagnosed formalin-fixed, paraffin-embedded (FFPE) classic HL specimens. Without microdissection, clonality was detected in 23 of 47 assessable cases. The IGK assay was significantly more sensitive than the IGH assay (18 vs 10 positive results). These data and 2 representative cases demonstrate that PCR-based B-cell clonality assays have usefulness when the histologic differential diagnosis of an FFPE specimen includes classic HL.

The diagnosis of Hodgkin lymphoma (HL) may become difficult owing to factors such as extranodal manifestation, small biopsy specimen, or histologic similarity to T-cell lymphoma. When the differential diagnosis includes nonhematopoietic and T-cell malignancies, robust methods to detect B-cell clonality may be useful because the neoplastic cells in HL have been demonstrated to be clonal B lymphocytes.1 A range of immunohistochemical markers, especially PAX5 and CD20, can help to distinguish HL from T-cell and nonhematopoietic processes. However, some cases remain challenging, particularly when the site is atypical, the clinical course is complex, or the specimen is in some way suboptimal. HL often manifests in deep mediastinal nodes, which are frequently difficult to excise, and specimens obtained by mediastinoscopy are small and fragmented, making evaluation of architecture challenging. Atypical manifestations of HL, in which lymphoma was not in the differential diagnosis at the time of biopsy, present the additional challenge that fresh tissue frequently is not available, and all additional studies must be performed on formalin-fixed, paraffin-embedded (FFPE) specimens.

The BIOMED-2 consortium effort has produced a set of polymerase chain reaction (PCR)-based methods for evaluating B-cell clonality and validated these extensively on fresh specimens of non-Hodgkin lymphomas (NHLs).2 The application of these assays to HL has been less extensively characterized.3,4 Furthermore, validation studies for these assays applied to FFPE specimens are limited. In current clinical practice, the majority of cases for which this type of ancillary study is required are likely to be FFPE specimens.

We assessed the usefulness of the BIOMED-2 IGH and IGK assays applied to FFPE HL specimens. We report our
findings and 2 cases as illustrative of the role that molecular diagnostic studies can fulfill in problematic HL cases.

Materials and Methods

Specimens
We retrieved 50 consecutive cases diagnosed as a classical-type HL (CHL) from the archive of surgical pathology at Strong Memorial Hospital, Rochester, NY, under a protocol approved by the research subject review board. The specimens were from the 2005-2007 period. Only cases with a definitive diagnosis of a CHL were selected for review; cases diagnosed as “suspicious,” “consistent,” “suggestive,” or with any other qualifier were not retrieved. None of the cases had a reported positive IGH clonality assay that may have contributed to the diagnosis. Flow cytometric results were reported for 44 cases; review of these data did not detect a clonal B-cell population in any case. Diagnoses were confirmed by review of histologic and immunohistochemical studies; additional immunohistochemical stains were performed as needed. Three specimens were rejected from further analysis because there was too little material in the paraffin blocks for DNA extraction and/or for additional immunohistochemical studies (CD20/79a). Of 47 cases, 6 had detectable expression of CD20 on the Reed-Sternberg (RS) cells, ranging from about 5% to 100% of the RS cells; the fraction of CD20+ cases (13%, with 95% confidence interval 6%-25%) is comparable to that generally reported. However, in practice, the number of CD79a+ cells was not in any case substantially different from the number of CD20+ cells. For B-cell and plasma-cell and CD30+ cell quantification, 10 high-power fields (HPF) were selected randomly, except that fields with more than 50% fibrosis were excluded for the CD30 enumeration. The percentages of B cells and plasma cells were rounded to the closest increment of 5. In the case of core biopsy specimens, the fields were selected linearly along the cores.

Immunohistochemical Staining
The estimated fraction of benign B cells and plasma cells in the specimen was based on CD20, PAX5, and/or CD79a stains. A CD79a stain was performed on selected cases in which the plasma cell number appeared substantial. However, in practice, the number of CD79a+ cells was not in any case substantially different from the number of CD20+ cells. For B-cell and plasma-cell and CD30+ cell quantification, 10 high-power fields (HPF) were selected randomly, except that fields with more than 50% fibrosis were excluded for the CD30 enumeration. The percentages of B cells and plasma cells were rounded to the closest increment of 5. In the case of core biopsy specimens, the fields were selected linearly along the cores.

DNA Preparation and PCR
DNA was isolated from four 10-μm sections of FFPE tissue using the QIAamp system (Qiagen, Valencia, CA). The concentration of DNA was estimated by spectrophotometry using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). DNA quality was assessed using a multiplexed control PCR ladder with amplicons of 100, 200, 300, 400, and 600 base pairs. Of 47 specimens, 44 showed amplification of the 400-base-pair control product. No specimen was excluded based on inadequacy of total DNA or the results of the control amplification.

PCR of the IGH and IGK genes to assess clonality used the BIOMED-2 system. Master mixes were purchased from Invivoscribe Technologies (San Diego, CA), and the PCR was done per manufacturer instructions and used HotStar Taq DNA polymerase (Qiagen). In an initial testing phase, we assessed reactions using the IGH FR2 and FR3 primer sets and both IGK primer sets. In a second phase, we assessed reactions using the IGH FR1 and D-J assays. The reaction was cycled 35 times between 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 90 seconds, preceded by 10 minutes at 94°C, and followed by 5 minutes at 72°C. After PCR, the products were heteroduplexed by raising the temperature to 95°C for 4 minutes followed by 2°C for 2 to 20 hours. Immediately after the heteroduplex step, the amplicons were resolved by electrophoresis on a preformed 10% polyacrylamide minigel in TBE buffer (Invitrogen, Carlsbad, CA) together with molecular weight markers (MspI digest of pUC18 plasmid DNA, Sigma, St Louis, MO), stained with SYBR Gold (Invitrogen), and photographed. Polyclonal (IVS-0000) and clonal (IVS-0007) control DNAs were purchased from Invivoscribe. Sensitivity of the assays was assessed using mixtures of clonal and polyclonal DNA. Products from 12 of the 18 positive IGK assays were purified and sequenced using the Jκ consensus primer. In each case, the sequence reflected rearrangement of the IGK locus (data not shown).

Results
Of 47 FFPE HL specimens, 23 had a clonal IGH and/or IGK result. Of 47 FFPE HL specimens, 23 had a clonal IGH and/or IGK result. The IGK assays contributed significantly more positive results than the IGH assays (18 vs 10 positive results; P = .03; 2-tailed Fisher exact test). Of the 10 cases with detectable IGH rearrangements, 9 were detected...
with assays of the FR2 and/or FR3 regions. The FR1 assay did not detect clonality in any specimen. The IGH D-J assay detected clonality in 2 cases; these cases had demonstrable clonality with the IGH FR2, IGH FR3, or IGK assays. Therefore, the IGH FR1 and D-J assays did not independently contribute to the detection of clonality in any case.

The IGH and IGK methods differed in their sensitivities to the number of RS cells and mononuclear variants. We estimated the number of RS cells and mononuclear variants by counting the average number of large CD30+ cells per high-power field [Table 2]. Cases were divided into 3 groups with increasing numbers of CD30+ cells. There seemed to be an association between the number of CD30+ cells and clonality detection using the IGH assay ($P = .05$; $\chi^2$). No evidence of a similar trend was seen for the IGK assay ($P = .5$). These findings suggest that clonality detection using the IGH assay is less dependent than the IGK assay on the number of CD30+ cells.

The effect of nonmalignant B cells and plasma cells was assessed by estimation of the fraction of cells staining for CD20 and/or CD79a [Table 2]. Cases were divided into 3 groups with increasing numbers of CD30+ cells. There seemed to be an association between the number of CD30+ cells and clonality detection using the IGH assay ($P = .05$; $\chi^2$). No evidence of a similar trend was seen for the IGK assay ($P = .5$). These findings suggest that clonality detection using the IGH assay is less dependent than the IGK assay on the number of CD30+ cells.

Although there seemed to be a relationship between expression of CD20 on the RS cells and the detection of clonality with any PCR primer set, the association did not reach significance ($P = .08$; 2-tailed Fisher exact test). Of the 47 cases, 6 (13%) had detectable expression of CD20 on RS cells: 5 of 6 CD20+ cases had detectable clonality; and 17 of 41 CD20− cases had detectable clonality.

Index Case 1

A 44-year-old man with no significant preexisting conditions underwent evaluation because of a several-month history of right thigh pain and then 1-week history of a readily apparent mass. Magnetic resonance imaging results were interpreted to show a heterogeneous soft tissue mass lying mostly in the vasti muscles and extending at least 25 cm distally from the femoral head. Involvement of the femur was also noted, suggesting a likely diagnosis of a soft tissue or bone sarcoma. Core biopsy specimens were obtained using a 16-gauge needle.

The lesion was composed predominantly of histiocytes, with numerous eosinophils, lymphocytes, and plasma cells [Image 1]. In addition to malignancy, the differential diagnosis for this small core biopsy specimen included peritumoral inflammation. Scattered through the lesion were some highly atypical cells with large nuclei with prominent nucleoli, some of which were eosinophilic. Occasionally these cells were clustered. The atypical cells were positive for CD30 and negative for other markers, including B-cell markers CD20, CD79a, and PAX5; T-cell markers CD2, CD3, CD4, and CD8; and macrophage/histiocyte markers CD68 and CD1a. CD15 and S-100 were also noted, suggesting a likely diagnosis of a soft tissue or bone sarcoma. Core biopsy specimens were obtained using a 16-gauge needle.

Radiographic studies indicated involvement of inguinal nodes, the patient’s disease was staged as IIEA, and therapy with doxorubicin [Adriamycin], bleomycin, vinblastine, and dacarbazine (ABVD) and local irradiation was started. The mass and pain resolved with therapy. Therapy was complicated by the need to place an intrafemoral rod. Six months

**Table 2**

<table>
<thead>
<tr>
<th>CD30+/HPF</th>
<th>Number</th>
<th>IGH+</th>
<th>IGK+</th>
<th>Clonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>14</td>
<td>0 (0)</td>
<td>4 (29)</td>
<td>4 (29)</td>
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<tr>
<td>11-30</td>
<td>14</td>
<td>5 (36)</td>
<td>5 (36)</td>
<td>8 (57)</td>
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<tr>
<td>&gt;30</td>
<td>19</td>
<td>5 (26)</td>
<td>9 (47)</td>
<td>11 (58)</td>
</tr>
</tbody>
</table>

HPF, high-power field; PCR, polymerase chain reaction.

* Data are given as number (percentage) unless otherwise indicated.

For IGH, $P = .05$; for IGK, $P = .5$; for IGH and IGK combined, $P = .2$ ($\chi^2$).

† Clonality detected by IGH and/or IGK PCR.
after completion of therapy, the patient was free of symp-
toms and positron emission tomography–computed tomo-
graphy scanning showed no evidence of recurrent disease.

Index Case 2

A 68-year-old woman was evaluated because of short-
ness of breath and cough. Radiology showed a pleural
effusion and soft tissue mass. The radiologic impression
was bronchogenic carcinoma. A transbronchial core needle
biopsy was performed. The biopsy showed histologic fea-
tures suggestive of HL, but the specimen was inadequate
for a definitive diagnosis. An open procedure was per-
formed that showed sheets of CD30+ large cells

Image 4. These cells lacked expression of CD15, CD20, CD79a,
and CD45. PAX5 reactivity was equivocal, while in some
areas, there was a suggestion of CD2 expression. Anaplastic
lymphoma kinase-1 and other T-cell markers were negative.
BIOMED-2 clonality assays for the T-cell receptor γ (data
not shown) and IGH (Image 2) loci were negative. A clonal
B-cell population was demonstrated using PCR of the IGK
locus (Image 3). A diagnosis of HL was made.

The disease was initially responsive to ABVD with
regression of the mediastinal mass. However, a lesion in the
right upper lobe expanded, and biopsy showed HL.

Discussion

Detection of clonality using PCR of the antigen recep-
tor genes has a relatively infrequent but occasionally critical
niche in the diagnostic workup of NHLs. The application

Image 11 (Index Case 1) A core biopsy of a soft tissue mass shows a proliferation of lymphocytes and histiocytes intermixed
with fibrosis (A, H&E, ×40). In a few regions, CD30+ mononuclear forms are abundant (B, ×200). A very rare form compatible
with a Reed-Sternberg cell is seen (C, H&E, ×400).

Image 21 Polymerase chain reaction analysis of the IGH
locus. The patient lanes (cases 1 and 2) were done in
duplicate. The upstream primers are from the FR2 or
FR3 region as indicated (BIOMED-2 IGH tubes B and C,
respectively), and the downstream primer is from the J
segment. The controls are polyclonal (normal lymphocyte),
10% + (10% monoclonal + 90% polyclonal), and HL60 (cell
line DNA lacking IGH rearrangement). The result for case 1
shows polyclonal B lymphocytes, and the result for case 2
shows no direct evidence for clonality but very little signal.
of PCR-based clonality detection for HLs is much less developed, but its role could be more critical than it is for NHL because, in contrast with the workup for NHL, flow cytometry does not detect the clonality in HL. Herein we show that about half of all CHL FFPE specimens can be expected to show B-cell clonality using a combination of the BIOMED-2 IGK and IGH assays.

The 2 cases presented herein indicate scenarios in which detection of clonality using DNA purified from FFPE tissue contributed substantially to the diagnosis. In the first case,

![Image 3](A) Polymerase chain reaction analysis of the IGK locus. The patient lanes (cases 1 and 2) were done in duplicate. The primer sets were designed to detect V-J joinings (BIOMED-2 IGK tube A; A) and IGK deletion (BIOMED-2 IGK tube B; B). The controls are the same as described in the legend for Image 2. The main clonal bands are indicated with arrows.

![Image 4](A) (Index Case 2) An excisional biopsy shows sheets of highly pleomorphic cells, with the same representative field shown at 2 magnifications (A, H&E, ×200; B, H&E, ×600). These cells are uniformly positive for CD30 (C, ×200).
the diagnosis was unsuspected owing to the unusual manifestation and strong clinical suspicion of a sarcoma. The infra-diaphragmatic manifestation and direct bone involvement are quite unusual sites of de novo HL. In the second case, the diagnosis was made difficult by the small specimen, the syncytial malignant cells that mimicked an NHL, and the lack of classic immunophenotype.

Only a handful of articles have described PCR-based clonality detection for HL using nonmicrodissected specimens. Just 3 of these assessed the use of the standardized BIOMED-2 primer system for FFPE HL specimens. The sensitivity for detecting clonality in HL in all of these studies is similar and clearly lower than that reported for most other histologic subtypes of lymphoma. There are several reasons that the PCR-based methods are less sensitive for HL compared with NHL. First, the malignant cells in HL are often relatively sparse. Second, there may be an abundance of nonmalignant B cells that produce a polyclonal background signal that lowers the analytic sensitivity for detection of the clonal signal. Third, the RS cells seem to subject their immunoglobulin loci to a high degree of somatic hypermutation, increasing the probability that one or more primer binding sites will be altered, causing the PCR to fail. Nevertheless, several groups have shown the successful application of the PCR-based BIOMED-2 clonality assays to HL (Table 4).

The sensitivity of the BIOMED-2 assays depends on the histology, the specimen type, and the specific assay. For example, the BIOMED-2 IGH assays have very high sensitivities when applied to small lymphocytic lymphoma specimens, whereas these specimens are fresh-frozen or FFPE. In contrast, the same assays fail for about 50% of FFPE follicular lymphoma (FL) specimens while showing more than 90% sensitivity for fresh FL specimens. The dependence on the choice of immunoglobulin locus is also exemplified by FFPE FL specimens: while the sensitivity is about 50% with IGH assays, the IGK assays have a sensitivity of more than 90%.

Although several recent articles on antigen receptor PCR in HL used the BIOMED-2 primer sets, our study differed in using heteroduplex analysis on polyacrylamide gels rather than Genescan analysis, which involves the use of a fluorescent primer and separation by capillary electrophoresis. Both methods were described in the initial BIOMED-2 publication and were reported to have similar sensitivities for the IGH and IGH PCR assays.

In the heteroduplex procedure, the PCR products are denatured and then allowed to reanneal. The likelihood of any specific DNA product reassociating with its exact complement in a polyclonal pool is extremely small. Therefore, the products derived from polyclonal B cells form a heteroduplex in which there are a small number of mismatches. The mismatches cause the annealed products to have a lower mobility in polyacrylamide gel electrophoresis. The specific amplicon from a clonal B-cell population is much more abundant and reassociates to form homoduplexes without mismatches. The heteroduplex signal derived from the polyclonal B cells appears at a higher apparent molecular weight, easing the visualization of the signal derived from the clonal population. This is shown in Image 5, which compares the results with and without the heteroduplex procedure for IGK PCR. Without the use of the heteroduplex procedure a small clone (10% and 5%) could not be seen in the strong polyclonal signal. When using the heteroduplex procedure, the clone is easily seen.

Laboratories that have compared the Genescan and heteroduplex methods have noted a preference for heteroduplexing in analysis of the IGK locus owing to its restricted junctional diversity, in contrast with a slight advantage in sensitivity for the Genescan method for the other antigen receptor genes. For HL, we found a similar overall clinical sensitivity compared with the other studies (Table 4) and concluded that the heteroduplex analysis provides an adequate and cost-effective alternative to approaches based on capillary electrophoresis.

Chute et al studied clonality in FFPE HL specimens using the BIOMED-2 IGH assays and found about 25% of HL cases to be clonal. These investigators found that the likelihood of clonality detection with the IGH assay increased with greater numbers of CD30+ cells. We also

<table>
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<th>Table 4</th>
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<td><strong>Summary of Published Studies of Clonality in Hodgkin Lymphoma Using the BIOMED-2 Method Applied to Formalin-Fixed, Paraffin-Embedded Specimens</strong></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Clonality</th>
<th>IGH</th>
<th>IGK</th>
<th>Total (%)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hebeda et al (n = 8)</td>
<td>3</td>
<td>5</td>
<td>6 (75)</td>
<td>40-93</td>
</tr>
<tr>
<td>McClure et al (n = 4)</td>
<td>2</td>
<td>2</td>
<td>2 (50)</td>
<td>14-85</td>
</tr>
<tr>
<td>Chute et al (n = 42)</td>
<td>10</td>
<td>ND</td>
<td>10 (24)</td>
<td>14-39</td>
</tr>
<tr>
<td>Present study (n = 47)</td>
<td>10</td>
<td>18</td>
<td>23 (49)</td>
<td>35-62</td>
</tr>
</tbody>
</table>

ND, not done.
We believe that this difference is large enough to explain the disparity. We suspect that this difference may be explained by the heteroduplex step in our procedure that reduces interference from the background signal due to polyclonal B cells. As shown in Image 5, the heteroduplex method allows clonal populations as small as 5% to be clearly detected in a polyclonal background.

Our finding that PCR can demonstrate clonality in about 50% of CHLs without microdissection is unlikely to be explained by “pseudoclonality.” First, sequencing of the IGK PCR products showed that all represent bona fide rearrangements of the IGK locus rather than spurious nonspecific reaction products. Second, all reactions were in duplicate and gave identical clonality results, precluding false-positives due to random mispriming in an early PCR cycle. Third, flow cytometry was performed on 20 of 23 CHL specimens positive for B-cell clonality; no suggestion of B-cell clonality was detected in any of these 20 specimens, indicating that a “composite” lymphoma is unlikely in any of these cases.

The BIOMED-2 IGK assays may be generally more robust for clonality detection in FFPE samples from malignancies in which the IGH loci are more heavily affected by somatic hypermutation, such as FL and HL. Our data showing the relatively greater robustness of the IGK compared with the IGH assay for HL are compatible with those recently suggested in a smaller study of 8 specimens (Table 4). We previously showed that the IGH assays failed in about 50% of FFPE FL specimens in which the IGK assays succeeded. Although the IGK assays are appreciably more sensitive, the IGH FR2 and FR3 assays contribute substantially to the overall sensitivity for CHL FFPE specimens. Thus, a clinical laboratory will need the IGK and IGH FR2/3 assays to achieve the best sensitivity in the evaluation of the occasional specimen of CHL that does not show typical clinical or histologic features.


