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Branched-Chain in situ Hybridization for κ and λ Light Chains: A Powerful Ancillary Technique for Determining B-Cell Clonality in Cytology Samples

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Abstract

BACKGROUND—Current immunohistochemical and in situ hybridization (ISH) assays are generally inconclusive for clonality unless plasmacytic differentiation is present. This study examined a series of cytology specimens and explored the ability of a branched-chain RNA (bRNA) ISH assay for immunoglobulin κ constant (*IGKC*) and immunoglobulin λ constant (*IGLC*) to detect a clonal population of B lymphocytes.

METHODS—Pathology databases were used to identify fine-needle aspiration biopsies (n = 28) and exfoliative cytology samples (n = 20). Demographic, flow cytometry, and excision biopsy results were recorded. bRNA ISH was performed on the Leica Bond platform with the following probes: *IGKC*, *IGLC*, immunoglobulin λ -like polypeptide 5 (*IgLL5*), and a housekeeping gene (HKG).

RESULTS—The bRNA ISH assay was validated with 30 surgical biopsies. On bRNA ISH, a clonal B-cell population (light-chain ratio > 10:1) was detected in 22 of 28 cases with a final diagnosis of lymphoma. In 2 cases, a κ predominance was present, although the ratio was <10:1. Eleven of the 17 κ -clonal lymphomas also expressed *IgLL5*, the latter recognized by the presence of an intranuclear signal. Two B-cell lymphomas lacked *IGKC* and *IGLC*, whereas 2 cases were negative for the HKG. In 12 of the 20 cases with reactive lymphoid tissue, bRNA ISH identified a

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CONFLICT OF INTEREST DISCLOSURES

Manoj Gandhi has a pending patent application entitled “Diagnosis of Multiple Myeloma and Lymphoma.” Miguel Rivera reports a sponsored research agreement with Affymetrix; in addition, Rivera has a patent pending for κ and λ in situ hybridization. David Ting reports a sponsored research agreement with Affymetrix; in addition, Ting has a patent pending for κ and λ in situ hybridization. Vikram Deshpande reports research support from Affymetrix during the conduct of the study; in addition, Deshpande has a patent pending for κ and λ in situ hybridization.

polyclonal lymphoid population. No light-chain messenger RNA was detected in 6 cases (typically those associated with very few B cells).

CONCLUSIONS—The automated bRNA ISH platform is a robust technique for detecting a clonal B-cell population in cytology material.

Keywords

B-cell lymphoma; immunoglobulin λ constant (*IGLC*); immunoglobulin κ constant (*IGKC*); immunoglobulin λ -like polypeptide 5 (*IGLL5*); in situ hybridization (ISH)

INTRODUCTION

Fine-needle aspiration cytology of lymph nodes is widely practiced, although the efficacy of this technique for the primary diagnosis of lymphoma remains controversial.^{1,2}

Nevertheless, in conjunction with flow cytometry, fine-needle aspiration biopsy may confirm the diagnosis of lymphoma and, in select cases, accurately classify the neoplasm and obviate surgical biopsy.^{3–5} The identification of a clonal population of lymphocytes, a task generally performed with flow cytometry, is an essential and occasionally indispensable ancillary test.⁶ In a proportion of cases, material for flow cytometry is unavailable, or the aspirate lacks the necessary cellularity; this frustrates the cytopathologist as well as the oncologist. Furthermore, some lymphoma cells are fragile and do not survive flow cytometry, lack surface light-chain expression, or show minimal surface light-chain expression; this precludes a determination of clonality. Finally, at sites such as the pancreas and liver, the diagnosis is often unsuspected, and material is not available for flow cytometry analysis. Although the specificity and sensitivity of cytology are relatively high, false-negative results are seen most often when flow cytometry is unavailable.⁷ Other, albeit less commonly used means of detecting B-cell clonality include immunoglobulin heavy chain (IGH) gene rearrangements and split-signal IGH DNA fluorescence in situ hybridization (FISH), although the latter is effective only for tumors with rearrangement at the IGH locus.⁸

Theoretically, stains for the expression of immunoglobulin light chains κ (immunoglobulin κ constant [*IGKC*]) and λ (immunoglobulin λ constant [*IGLC*]) would be able to distinguish a polyclonal population of lymphocytes from a monoclonal one. Although conventional methods for detecting clonal B-cell populations such as immunohistochemistry and in situ hybridization (ISH) readily detect light chains in plasmacytomas as well as lymphomas with plasmacytic differentiation such as mucosa-associated lymphoid tissue (MALT) lymphomas, these techniques are generally unsuccessful with cytology preparations, primarily because of issues related to nonspecific staining as well as an inability to detect low-level protein and messenger RNA (mRNA) expression in the majority of B-cell lymphomas.

The availability of a branched-chain RNA (bRNA) ISH assay, a robust means of detecting mRNA in paraffin-embedded tissue, has the potential to transform current clinical practice.^{9,10} The increased sensitivity of this assay relies on signal amplification, and it has the potential to detect single-copy RNA transcripts.^{11,12} The assay detects light-chain mRNA in mature lymphocytes: conventional ISH techniques are seldom successful at

detecting low-level mRNA expression in lymphocytes. Theoretically, the bRNA ISH platform could provide a robust means of assessing clonality.

However, the interpretation of this assay is associated with some unique challenges: κ light chain–positive B-cell lymphomas also express a surrogate λ light chain—immunoglobulin λ -like polypeptide 5 (*IGLL5*)—and as a result, κ light chain–producing lymphomas appear to be positive for both κ and λ light chains.¹² This apparent coexpression of light chains could potentially erase the benefits associated with the high sensitivity of the bRNA ISH assay.

In this study, we examined fine-needle aspirates and exfoliative cytology samples and explored the ability of the bRNA ISH assay for *IGKC* and *IGLC* to detect B-cell clonality. We also evaluated an interpretive algorithm that facilitates the distinction of *IGLC* from *IGLL5*.

MATERIALS AND METHODS

This study was approved by the institutional review board at the Massachusetts General Hospital.

Study Cases

Clinical and pathology databases were used to identify fine-needle aspiration biopsies (n = 28) and exfoliative cytology samples (n = 20) evaluated at the Massachusetts General Hospital between January 2011 and December 2014. Inclusionary criterion included the presence of a prominent lymphoid population as well as a cell block preparation with at least 100 lymphocytes. Flow cytometry data were available in the majority of cases. Reactive lymphoid lesions, B-cell lymphomas, and 2 plasmacytic neoplasms were included in the study. The final diagnosis and subtyping were based on excisional biopsies in the majority of cases. The diagnosis of benign lymphoid proliferation was based on a combination of excisional biopsies, flow cytometry (when available), and clinical follow-up.

In an attempt to assess the validity of the probes, paraffin sections from 30 excisional biopsies were also evaluated. The cohort was composed of 21 B-cell lymphomas and 9 reactive lymph nodes. The excisional biopsies were independent of the fine-needle and exfoliative cytology samples. bRNA ISH was performed on the cytology samples as well as excisional biopsies.

Automated κ and λ Light-Chain RNA ISH Assay

All 48 cases were assayed with a commercially available automated RNA ISH platform for *IGKC* (catalog number ZVA1-15950), *IGLC* (catalog number ZVA1-15951), and *IGLL5* ViewRNA eZ probes (catalog number DVA1-16581; Affymetrix, Santa Clara, Calif). The RNA integrity of the specimens was determined with a cocktail of glyceraldehyde 3-phosphate dehydrogenase, β -actin, and a peptidylprolyl isomerase B ViewRNA eZ probe (catalog number DVA1-16742), which served as a housekeeping gene (HKG). Automated ISH assays were performed with the ViewRNA eZ-L detection kit (catalog number 19500; Affymetrix) on the Bond RX automated immunohistochemistry and ISH staining system

with BDZ 6.0 software (Leica Biosystems, Buffalo Grove, Ill). Sections from formalin-fixed, paraffin-embedded cell block preparations were processed automatically from deparaffinization through ISH staining to hematoxylin counterstaining. Briefly, 5- μ m-thick sections were baked for 1 hour at 60°C and placed on the Bond RX for processing. The Bond RX user-selectable settings were as follows: ViewRNA 1 protocol, ViewRNA Dewax 1, ViewRNA HIER for 10 minutes and ER2 (90), ViewRNA Enzyme 2 for 7 minutes, and ViewRNA probe hybridization for 3 hours. With these settings, the RNA unmasking conditions for the cytology cell block sections consisted of a 10-minute incubation at 90°C in Bond Epitope Retrieval Solution 2 (Leica Biosystems) followed by a 7-minute incubation with proteinase K from the Bond enzyme pretreatment kit at a 1:1000 dilution (Leica Biosystems). *IGKC* and *IGLC* probes (Affymetrix) were diluted (1:10) in the ViewRNA probe diluent (Affymetrix). The pooled HKG probe was diluted (1:40) in the ViewRNA probe diluent (Affymetrix). Posthybridized slides were rinsed with water, air-dried for 30 minutes at room temperature, mounted with Histomount organic mounting media, and visualized with a standard bright-field microscope with 20 \times and 40 \times objectives. Positive ISH staining was defined as punctate red-colored dots present in the nucleus or cytoplasm of lymphocytes. Additional details on automation are provided at the Panomics Web site.¹³

A 2-color assay (n = 10) was performed for select cases in a manual format as described in a prior publication.^{9,10} The assay allowed the simultaneous detections of *IGLC* and *IGKC* on a single slide.¹⁰ The probes were Type 1 *IGKC* (catalog number VA1-11526) and Type 6 *IGLC* (catalog number VA6-13793; Affymetrix); the dilution was 1:20.

Flow Cytometry

Eight-color flow cytometry analyses were performed as previously described in a publication from this institution.¹⁴

Immunohistochemistry and Conventional ISH

The source of the antibodies and ISH probes and the antigen retrieval conditions are listed in Table 1. Immunohistochemistry for paired box 5 (PAX5) was performed for a subset of cases (n = 24), including all cases negative for κ and λ light chains. Immunohistochemistry and conventional ISH were performed for select cytology samples: immunohistochemistry for κ and λ light chains was performed in 16 cases, and conventional ISH for κ and λ light chains was performed in 19 cases. Immunohistochemistry for κ and λ light chains was performed for all excision specimens.

RESULTS

Paraffin-Embedded Sections From Lymph Node Excisions

To validate the bRNA ISH technology, we evaluated paraffin-embedded sections from excised lymph nodes, including 21 lymphomas (11 diffuse large B-cell lymphomas, 6 follicular lymphomas, and 4 mantle cell lymphomas) and 9 reactive lymph nodes.

Robust staining was observed in plasma cells and lymphocytes, although the number of transcripts in mature lymphocytes was significantly lower than that in plasma cells. In

contrast, clonality could not be determined from immunohistochemical stains for κ and λ light chains. As determined by bRNA ISH, 14 cases showed clonal *IGKC*-positive B-cell populations, whereas 7 cases were composed of *IGLC*-positive B cells. The malignant follicles were composed of clonal populations of B cells in all 6 follicular lymphomas, whereas the mantle zone and interfollicular tissue was composed of polyclonal populations of lymphocytes and plasma cells (Fig. 1). Notably, all 14 cases of κ -clonal lymphomas also showed staining for the *IGLC* probe; the latter was predominantly intranuclear. The *IGLC* nuclear signal in κ -clonal lymphocytes was a reflection of the *IPLL5* transcripts and not *IGLC* mRNA; this finding was confirmed with a probe specific for *IPLL5*.

Six cases that were indeterminate on flow cytometry were identified to have light-chain restriction with bRNA ISH. In cases with measurable levels of surface or cytoplasmic light chains on flow cytometry, a perfect concordance between flow cytometry and bRNA ISH was achieved. In contrast, small lymphocytes in these samples were typically negative for light-chain expression with immunohistochemistry, but the plasma cells showed polytypic staining.

The 9 reactive lymph nodes showed a polyclonal population of *IGKC*-positive and *IGLC*-positive B cells. The germinal center and the mantle zone and interfollicular tissue were composed of polyclonal populations of lymphocytes and plasma cells. The T cell-rich zones were devoid of light-chain transcripts.

Cytology Samples

Aspirates from lymph nodes (n = 17) and pleural fluid (n = 14) were the 2 most common specimens evaluated in the study (Table 2). Fine-needle aspirates from other sites included the following: lung (n = 3), parotid gland (n = 3), pancreas (n = 2), subcutaneous soft tissue (n = 2), and stomach (n = 1). Other exfoliative samples included vitreous fluid (n = 3), ascitic fluid (n = 2), and pericardial fluid (n = 1).

Cases With a Final Diagnosis of Reactive Lymphoid Tissue (n = 20)

The mean age of this cohort was 57 years (range, 31–85 years) with 11 males and 9 females. The cytology smears and the corresponding cell block preparations showed mature lymphocytes. Plasma cells were observed in half of the cases (n = 9). The lymph node aspirates (n = 7) showed a predominantly small lymphocytic population: lymphoid tangles and tingible body macrophages were identified in 3 of the 7 lymph nodes. No atypical lymphoid cells were noted. On follow-up (median, 18 months), these cases did not progress to lymphoma.

Flow cytometry was performed for 15 of the 20 cases: 13 cases with detectable B cells and plasma cells were interpreted as polyclonal, and 2 cases showed insufficient B cells. In comparison, bRNA ISH identified 12 cases with polyclonal lymphocytes/plasma cells: the κ/λ ratio ranged from 2:1 to 4:1 (Fig. 2). No staining for κ or λ was observed in 6 cases; however, a few scattered B cells were identified on the Pax5 stain, and this suggested that not all mature lymphocytes showed detectable levels of light-chain mRNA. One case showed significant background staining, likely a result of poor RNA preservation, as determined by an evaluation of the HKG.

Case 16, originally interpreted as a reactive lymph node, showed a clonal κ population on bRNA ISH. A polymorphic population of lymphocytes was noted on the Giemsa-stained preparation, although the aspirate was devoid of tingible body macrophages (Fig. 3). Flow cytometry samples showed 4% B cells; however, the percentage of B cells as determined by the Pax5 stain was significantly higher (>80%). The patient was lost to follow-up, and thus the significance of this clonal B-cell population is uncertain.

Conventional ISH and immunohistochemistry for κ and λ , performed for 5 and 6 cases, respectively, failed to detect a sufficient signal within the lymphoid population to allow the determination of clonality.

Cases With a Final Diagnosis of a B-Cell Lymphoma (n = 28)

The mean age of this group was 67 years (range, 39–92 years) with 16 males and 12 females. Based on analyses of excision biopsies and immunohistochemical and flow cytometry data, the diagnoses were as follows: diffuse large B-cell lymphoma (n = 8), follicular lymphoma (n = 7), small lymphocytic lymphoma (n = 3), plasmacytoma (n = 2), Burkitt lymphoma (n = 2), mucosa-associated lymphoid tissue lymphoma (n = 1), and mantle cell lymphoma (n = 1). Four cases lacked both excisional biopsies and diagnostic features on flow cytometry and were classified as low-grade B-cell lymphomas.

In 22 cases, bRNA ISH showed a predominance of a single immunoglobulin light chain (ratio > 10:1; Fig. 4). Two cases showed κ predominance; however, the ratio was <10:1 but >4:1. Among the 17 κ -clonal lymphomas, cytoplasmic reactivity for *IGKC* in 11 cases was also accompanied by nuclear reactivity for *I_gLC* (Figs. 5 and 6). This pattern was interpreted as a clonal κ -positive population with coexpression of *I_gLL5*: nuclear *I_gLL5* reactivity was confirmed on ISH for *I_gLL5*. The 5 *I_gLC* clonal lymphomas lacked *I_gLL5* reactivity (Fig. 7). bRNA ISH was negative in 2 cases: PAX5 staining was positive, whereas the HKG confirmed the presence of preserved RNA. This suggested that these cells either lacked light-chain transcripts or were below the detectable limits. Two additional cases were negative for the HKG, and this suggested a lack of RNA preservation.

Flow cytometry detected a clonal lymphoid population in 17 of 21 cases. Two diffuse large B-cell lymphomas and 2 follicular lymphomas were negative for a clonal population of lymphocytes: bRNA ISH recognized light-chain restriction in 3 of the 4 cases.

Conventional ISH and immunohistochemistry for κ and λ were performed for 11 and 13 cases, respectively. Both techniques detected a clonal population in cases 35 and 37: plasma cell myeloma. In the other cases (all B-cell neoplasms), these conventional techniques failed to detect a clonal B-cell population.

DISCUSSION

Identifying a clonal B-cell population is a critical and often indispensable step in the diagnosis of B-cell lymphoma; this distinguishes a lymphoproliferative disease from reactive lymphoid proliferation.^{2,3,5,15,16} One exception to this rule is diffuse large B-cell lymphoma, which in most cases does not require clonality studies to establish a diagnosis. Most non-

Hodgkin lymphomas express low levels of *IGKC* and *IGLC* mRNA transcripts, and typically κ and λ light chains cannot be detected by immunohistochemistry or conventional ISH. The absence of tissue architecture on fine-needle aspiration and exfoliative cytology samples enhances this dependence on ancillary tests. For cytology specimens, flow cytometry is the most commonly used assay to assess clonality: the assay is sensitive and, in certain scenarios, obviates open biopsy, particularly at sites at which excisional biopsy may prove difficult. Commonly, fresh cells, a prerequisite for flow cytometry, may not be available, particularly when suspicion for a lymphoma is relatively low. It is notable that neither immunohistochemistry nor any other currently available automated ISH assay represents a robust means for detecting B-cell clonality in cases other than those with plasmacytic differentiation.

In the study, we have validated an automated bRNA ISH assay for κ and λ light chains on resected lymph nodes. We have also shown that this novel ISH assay distinguishes a monoclonal B-cell population from a polyclonal B-cell population in cytology preparations. The evaluation of a reactive lymphoid population was often further simplified by the relative paucity of B lymphocytes: several reactive lymphoid infiltrates showed only rare B lymphocytes, as indicated by rare PAX5-positive lymphocytes. As with conventional ISH assays, plasma cells were readily recognized as polyclonal. The majority of B-cell lymphomas were classified as monoclonal, and they included a diverse collection of B-cell lymphomas: diffuse large B-cell lymphoma, follicular lymphoma, small lymphocytic lymphoma, and Burkitt lymphoma. The bright cytoplasmic staining of mRNA transcripts allowed easy interpretation. However, a fairly wide range of light-chain expression was noted, with a minority of cases showing only a few transcripts. The increased distance between individual lymphocytes allowed a clear distinction of nuclear and cytoplasmic reactivity; cytology samples proved easier to interpret than resected lymph nodes.

Six cases (highlighted in bold in Table 2) that did not have a prior history of lymphoma and lacked tissue for flow cytometry provide a glimpse into the ability of the assay to redefine the current diagnostic algorithm. An aspirate from the vitreous fluid of a 67-year-old male (case 42) was interpreted as a reactive lymphoid infiltrate. The bRNA ISH stain revealed a κ -positive B-cell lymphocyte population. Flow cytometry analysis of a subsequent vitrectomy specimen confirmed the diagnosis of a B-cell lymphoma. The diagnosis of vitreoretinal lymphoma remains a diagnostic challenge, and the application of this sensitive platform to detect clonality is likely to facilitate the distinction of inflammatory diseases from B-cell lymphomas.¹⁷

One predictable interpretation difficulty in using this highly sensitive bRNA ISH assay is the presence of a λ -like pseudogene (*IGLL5*) in κ -producing lymphomas and within reactive germinal centers. The apparent coexpression of κ and λ in κ -positive lymphoma cells represents *IGLL5* and not λ light-chain mRNA transcripts. Interestingly, *IGLL5* is detected only in κ -producing lymphocytes and is undetectable in λ -positive lymphocytes. The *IGLL5* gene is located in the immunoglobulin λ locus (22q), but unlike the λ locus, it does not undergo somatic rearrangement. It should be noted that significant homology between *IGLC* and *IGLL5* precludes the development of a λ -specific probe. However, it is feasible to develop a probe specific for *IGLL5* because the first exon is unrelated to the λ locus. This

restriction of *IGLL5* to κ -producing lymphocytes was confirmed on a probe specific for *IGLL5*. Notably and in contrast to *IGLC*, *IGLL5* is localized predominantly to the nucleus. Moreover, the *IGLL5* intranuclear dots are significantly larger than *IGLC*; that is, larger intranuclear dots with the *IGLC* probe suggest *IGLL5* cross-reactivity and not λ reactivity (see the algorithm in Fig. 8).

The bRNA ISH assay provides a robust means of detecting a clonal population of B lymphocytes in cytology samples; however, some additional caveats apply, particularly for those with limited numbers of B lymphocytes. These samples may show an artifactually skewed κ/λ ratio. As a result, clonality as defined by RNA ISH requires the overwhelming dominance (>10:1) of a clone.

This study identified 1 significant outlier: case 16. Although the bRNA ISH identified a clonal population of lymphocytes, flow cytometry failed to detect a clonal population; however, the sample appeared to be composed predominantly of blood. Although the patient was lost to follow-up, the possibility that this patient harbored a lymphoproliferative disease could not be excluded. Clonal B-cell populations in nonlymphomatous processes have been rarely reported in reactive lymph nodes and have been mainly observed in patients with viral illnesses or auto-immune or immunodeficiency syndromes.^{18,19} However, because both polymerase chain reaction–based studies and flow cytometry are more sensitive, we predict that the incidence of these benign clonal populations on bRNA ISH is likely to be lower. Nevertheless, this case underscores the need for a consolidated and thorough review of all available morphological, immunohistochemical, ISH, flow cytometry, and molecular data before a diagnosis of a B-cell lymphoma is confirmed.

In conclusion, the automated bRNA ISH platform represents a robust means of distinguishing a monoclonal B-cell/plasma cell population from a polyclonal one in both fine-needle aspiration biopsies and exfoliative cytology samples. The automated version of the assay fits easily into the workflow of most modern clinical laboratories. Although this analysis was performed on paraffin-embedded material, the assay could also be adapted for use with alcohol or air-dried smears. One significant advantage of cell block–based testing is that subsequent sections can be used for immunohistochemistry, FISH-based assays, and molecular and next-generation sequencing.²⁰ Although *IGLL5* represents a confounding factor, nuclear localization generally allows for the recognition of this pseudogene. The 2-color assay would address, in part, this shortcoming: cells with dual reactivity (ie, red dots [*IGKC*] in the cytoplasm and blue dots [*IGLC*] in the nucleus) would identify *IGLL5*-bearing, κ -positive lymphocytes. However, although the single-color assay is currently automated, an automated 2-color assay is yet to be validated. Although flow cytometry is more sensitive than bRNA ISH, this assay is likely to be immensely helpful in several clinical scenarios: 1) material for flow cytometry is unavailable; 2) the sample for flow cytometry lacks adequate cellularity or is composed predominantly of blood; and 3) a way of validating flow cytometry data, particularly samples with equivocal results, is needed. The availability of a robust assay for detecting clonality in cell block preparations would bring cytology closer to the goal of averting open biopsy at inaccessible sites. This assay adds to the growing list of ancillary techniques, which include flow cytometry, polymerase chain reaction for B- and T-cell gene rearrangements, FISH, and next-generation sequencing, and

brings the field closer to the often stated goal of sparing patients from excisional biopsy in select cases.

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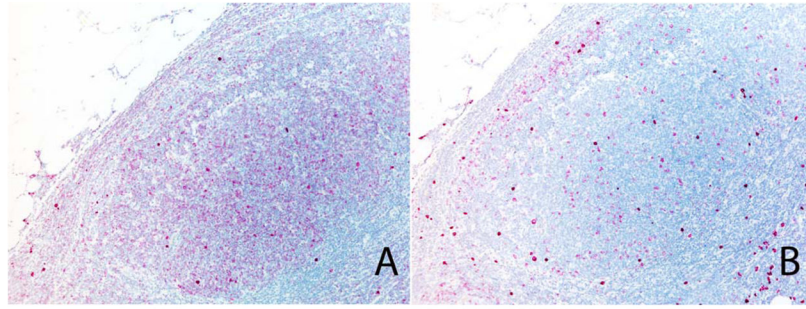


Figure 1. Branched-chain in situ hybridization: excisional biopsy. (A) This follicular lymphoma shows strong immunoglobulin λ constant reactivity in the follicles. (B) The immunoglobulin κ constant stain shows positive cells in the mantle zone (in situ hybridization assay).

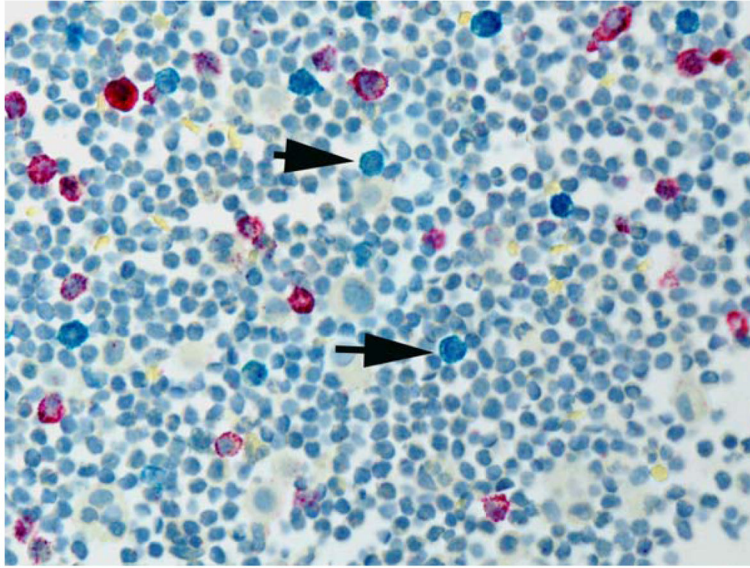


Figure 2. Two-color in situ hybridization assay performed on a pleural fluid with reactive lymphocytes and plasma cells. Both immunoglobulin κ constant-positive (red chromogen) and immunoglobulin λ constant-positive (blue chromogen) lymphocytes and plasma cells are present. The blue chromogen (arrow) is significantly less prominent than the red chromogen (in situ hybridization assay).

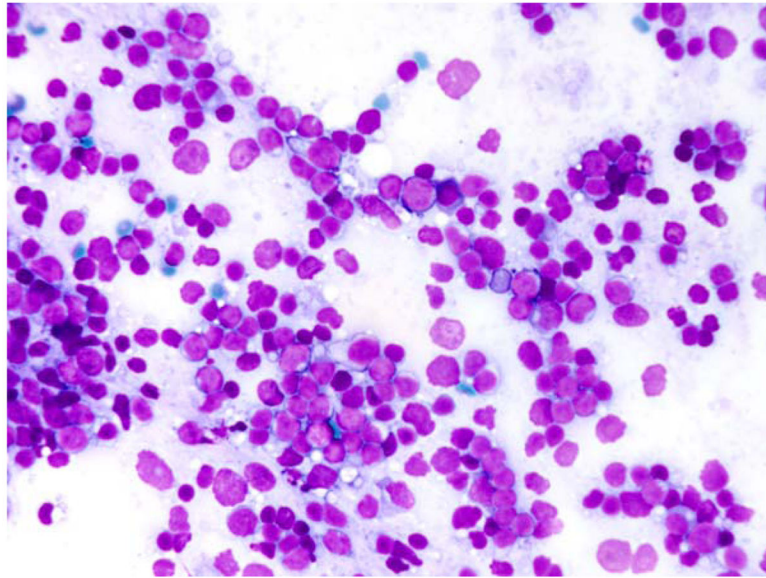


Figure 3. Case 16. This lymph node aspirate shows a polymorphic population of lymphocytes (May-Grünwald-Giemsa stain).

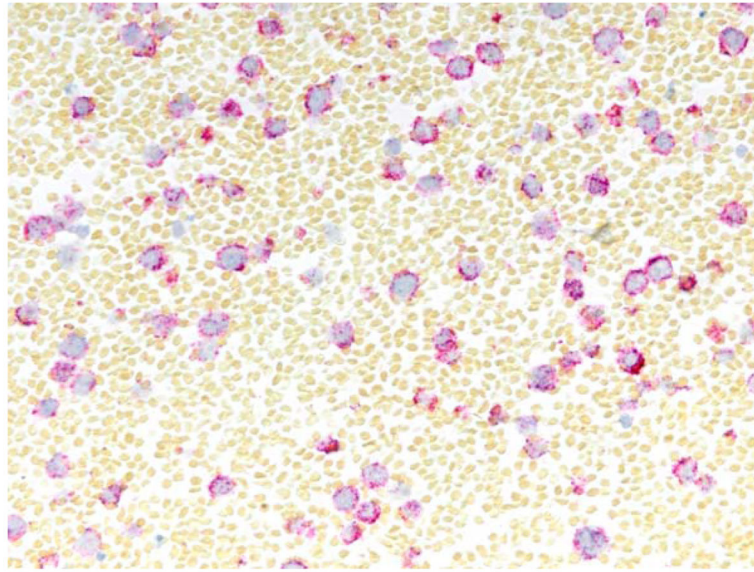


Figure 4. Two-color in situ hybridization performed on a diffuse large B-cell lymphoma positive for immunoglobulin κ constant (red chromogen) and supporting the diagnosis of κ -clonal proliferation. Immunoglobulin λ constant (blue chromogen) is not seen (2-color in situ hybridization assay).

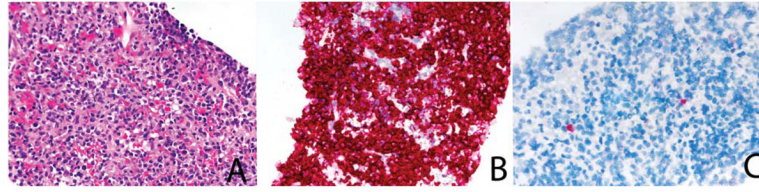


Figure 5. (A) Plasma cell myeloma (H & E) with (B) intense reactivity for immunoglobulin κ constant. (C) The immunoglobulin λ constant stain shows a single positive plasma cell (in situ hybridization assay).

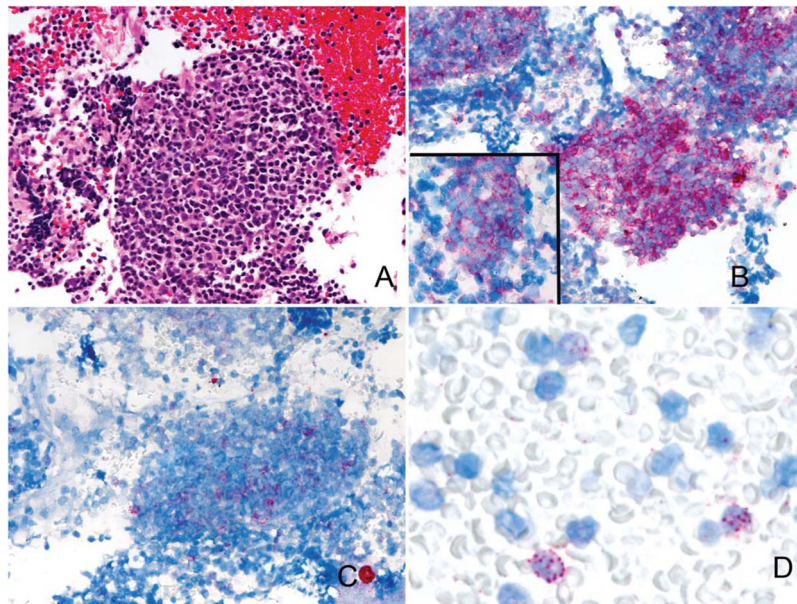


Figure 6. (A) Peripancreatic lymph node from a patient with a grade 1 follicular lymphoma (H & E). (B) The immunoglobulin κ constant stain is intensely positive, and a follicular pattern is appreciable. The negatively stained cells represent T cells (results not shown). (C) Occasional cells are positive on the immunoglobulin λ constant stain. (D) However, as shown at a high-power magnification, these cells show predominantly nuclear reactivity, a pattern typical of immunoglobulin λ -like polypeptide 5 reactivity (in situ hybridization assay).

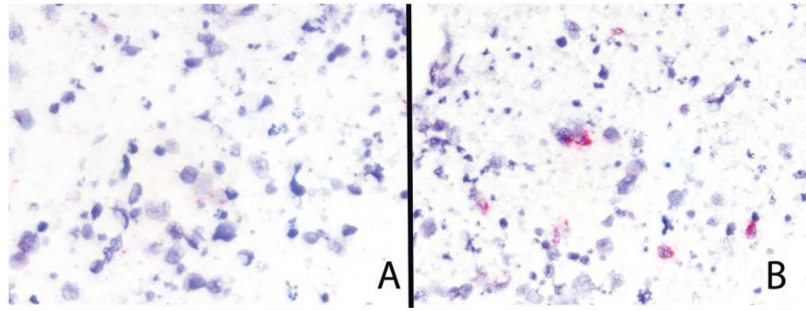


Figure 7. Mantle cell lymphoma. The tumor cells are relatively poorly preserved. (A) Immunoglobulin κ constant is negative. (B) Immunoglobulin λ constant shows occasional cells with strong reactivity confined to the cytoplasm of the tumor cells (in situ hybridization assay).

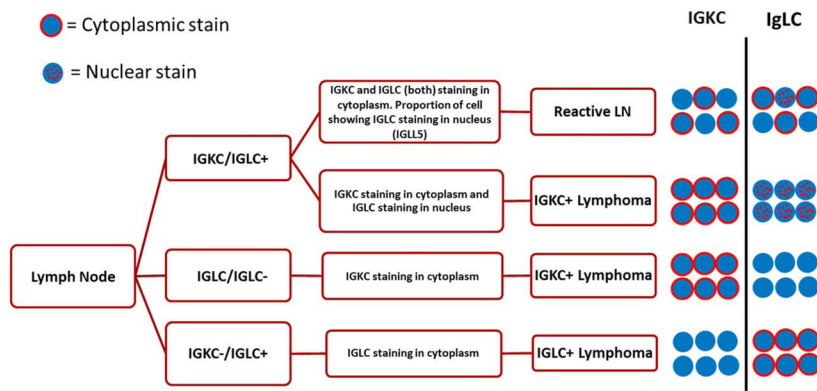


Figure 8. Algorithm for interpreting *IGLC* and *IGKC* in situ hybridization stains. Abbreviations: IGKC, immunoglobulin κ constant; IGLC, immunoglobulin λ constant; IGLL5, immunoglobulin λ -like polypeptide 5; LN, lymph node.

TABLE 1**IHC and Conventional ISH: Source of Antibody and Probes**

	Source	Catalog Number	Dilution	Antigen/RNA Retrieval
κ ISH (conventional)	Leica	5748a	Ready to use	Enzyme 1 for 15 min
λ ISH (conventional)	Leica	5770a	Ready to use	Enzyme 5 for 15 min
κ IHC	Leica	NCL-kap-581	1:100	ER1 (citrate at pH 6) for 20 min
λ IHC	Leica	NCL-L-LAM570	1:7000	ER1 for 10 min
PAX5 IHC	Leica	PA0552	Ready to use	ER2 (EDTA at pH 9) for 20 min

Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER1, Epitope Retrieval Buffer 1; ER2, Epitope Retrieval Buffer 2; IHC, immunohistochemistry; ISH, in situ hybridization; PAX5, paired box 5.

TABLE 2

Clinical, Flow Cytometry and ISH Results

Case No.	Age, y	Sex	Specimen Type	B-Cell Flow, %	Clonality on Flow	Final Diagnosis	Initial Diagnosis of Lymphoma	Clonal on bRNA ISH
1	36	Female	Lung	2	Polyclonal	Reactive	NA	Polyclonal
2	84	Male	Pleural fluid	0	No B cells	Reactive	NA	No B cells
3	47	Female	Pleural fluid	31	Polyclonal	Reactive	NA	No B cells
4	58	Male	Pleural fluid	2	Polyclonal	Reactive	NA	No B cells
5	45	Male	Lymph node, groin	ND	ND	Reactive	NA	Polyclonal
6	61	Male	Pleural fluid	8	Polyclonal	Reactive	NA	Polyclonal
7	63	Male	Ascitic fluid	22	Polyclonal	Reactive	NA	Polyclonal
8	52	Female	Pleural fluid	7	Polyclonal	Reactive	NA	Polyclonal
9	36	Female	Pleural fluid	2	Polyclonal	Reactive	NA	Polyclonal
10	31	Male	Lymph node	20	Polyclonal	Reactive	NA	Significant background
11	84	Male	Pleural fluid	0	No B cells	Reactive	NA	No B cells
12	31	Male	Lymph node, portal	ND	ND	Reactive	NA	Polyclonal
13	34	Male	Vitreous fluid	ND	ND	Reactive	NA	No B cells
14	40	Female	Lymph node, neck	12	Polyclonal	Reactive	NA	No B cells
15	75	Female	Lung	3	Polyclonal	Reactive	NA	Polyclonal
16	63	Male	Lymph node, neck	4	Polyclonal	Reactive	NA	Clonal κ
17	54	Male	Lymph node, neck	ND	ND	Reactive	NA	Polyclonal
18	70	Female	Lymph node, retroperitoneal	25	Polyclonal	Reactive	NA	Polyclonal
19	85	Female	Pleural fluid	ND	ND	Reactive	NA	Polyclonal
20	82	Female	Pleural fluid	20	Polyclonal	Reactive	NA	Polyclonal
21	85	Female	Pleural fluid	9	κ	Low-grade B-cell lymphoma	NA	Clonal κ
22	39	Male	Lymph node, neck	46.00	κ	Low-grade B-cell lymphoma	No	Clonal κ
23	48	Female	Lung	16	κ	Low-grade B-cell lymphoma	No	Clonal κ
24	82	Female	Soft tissue, paraspinal	51	κ	Low-grade B-cell lymphoma	No	Clonal κ
25	75	Female	Lymph node, neck	24	λ	Follicular lymphoma grade 2/3	Yes	Clonal λ
26	45	Male	Lymph node, peripancreatic	45	Inconclusive	Follicular lymphoma grade 1-2/3	Yes	Clonal κ
27	72	Male	Pancreas	52	Inconclusive	Follicular lymphoma, grade 1-2	No	No B cells
28	79	Male	Lymph node, abdominal	63	κ	Follicular lymphoma	No	Clonal κ

Case No.	Age, y	Sex	Specimen Type	B-Cell Flow, %	Clonality on Flow	Final Diagnosis	Initial Diagnosis of Lymphoma	Clonal on bRNA ISH
29	76	Male	Lymph node, abdominal	46	κ	Follicular lymphoma, grade 1	No	κ predominance
30	92	Female	Parotid	41	κ	Follicular lymphoma, grade 1-2	Yes	Clonal κ
31	67	Male	Lymph node, neck	28	λ	Follicular lymphoma	No	Clonal λ
32	62	Male	Lymph node, neck	71	λ	SLL	Yes	Clonal λ
33	68	Female	Parotid	40	κ	SLL	No	κ predominance
34	46	Male	Parotid	Inconclusive		SLL	No	Clonal κ
35	68	Female	Pleural fluid	ND	ND	Plasma cell myeloma	No	Clonal κ
36	72	Male	Stomach	ND	ND	MALT lymphoma	Yes	Clonal κ
37	65	Female	Subcutaneous, chest wall	16	λ	Plasma cell myeloma	Yes	Clonal λ
38	40	Female	Ascitic fluid	ND	ND	Burkitt	No	No B cells
39	79	Male	Pleural fluid	16	λ	Mantle cell lymphoma	No	Clonal λ
40	65	Male	Lymph node	83	κ	Burkitt	No	Clonal κ
41	85	Male	Pleural fluid	28	Inconclusive	DLBCL	No	Clonal κ
42	67	Male	Vitreous fluid	ND	ND	DLBCL	Yes	Clonal κ
43	61	Female	Lymph node, neck	26	κ	DLBCL	Yes	Clonal κ
44	86	Female	Vitreous fluid	ND	ND	DLBCL	Yes	Clonal κ
45	73	Female	Pancreas	ND	ND	DLBCL	Yes	Assay failed
46	69	Male	Pericardial	48	κ	DLBCL	No	Clonal κ
47	54	Male	Pleural fluid	2	Inconclusive	DLBCL	No	Clonal κ
48	66	Male	Lymph node, retroperitoneal	30	κ	DLBCL	Yes	Assay failed

Abbreviations: bRNA, branched-chain RNA; DLBCL, diffuse large B-cell lymphoma; ISH, in situ hybridization; MALT, mucosa-associated lymphoid tissue; NA, not available; ND, not done; SLL, small cell lymphoma. Bold font represents cases that do not have a prior history of lymphoma and lack tissue for flow cytometry.