Ancillary Testing in Lymphoma Diagnosis and the Challenges of Small Biopsies

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Objectives

• Review the utility and necessity of ancillary testing in the diagnosis of lymphoma.
• Discuss the use of FISH assays to provide prognostic information or precise classification in lymphoma diagnosis.
• Discuss the challenges and perils of small biopsies in lymphoma diagnosis.
Lymphoma: 86 types (+ subtypes)
How do we diagnose and classify lymphomas?

• Morphology forms the basis for the lymphoma classification system.
  » Architecture: nodal effacement vs. preservation?
    ▪ Diffuse, follicular, nodular
  » Cell characteristics: large vs. small, blast vs. mature.
  » Hodgkin vs. non-Hodgkin

• Role of ancillary testing continues to increase.
  » Immunophenotype by flow cytometry or immunohistochemistry.
    ▪ B-cell vs. T-cell, other characteristic antigen expression.
  » Molecular/genetic characteristics: translocations, mutations.
Ancillary Tests Used in Lymphoma Diagnosis

- Immunophenotyping
  - Flow cytometry and/or immunohistochemistry
- Cytogenetics
  - Karyotype or FISH for translocations
- Molecular
  - Clonality
  - PCR for specific translocations or mutations
  - Sequencing for mutations, translocations
Flow Cytometry

• Most sensitive and complete immunophenotyping.
  » Determine expression of multiple antigens on one cell population.
• Detect clonal B-cells
  » Polytypic: Mix of kappa and lambda expressing cells.
  » Monotypic: Predominant expression of one or the other.
• Detection of abnormal T-cell populations.
• Detect low-level involvement.
CLL/SLL: Flow Cytometry Does This Well

- Characteristic immunophenotype: CD5, dim CD20, dim monoclonal light chains, CD23, CD200.
  - Diagnostic in appropriate clinical setting.
  - Imperfect phenotypes should raise consideration of other entities, especially mantle cell lymphoma.

- Only way to characterize monoclonal B-cell lymphocytosis (MBL).
  - CLL type, atypical CLL type, non-CLL type.
  - Low-count (<0.5x10^9/L) does not seem to progress.
  - High-count (≥0.5-5x10^9/L) acts like Rai stage 0 CLL.
Limitations of Flow Cytometry

• Loss of morphologic features, which are key for the WHO classification system.
  » Generally cannot give a specific WHO diagnosis.
  » CD10 positive lymphoma, not “follicular lymphoma.”

• False negative: May lose some cell populations in processing, analysis, and/or sampling.
  » Lymphoma cells, especially large cells, may be excluded.
  » Sampling is critical.

• “False positive”: Small clonal B-cell populations and aberrant T-cell populations are not diagnostic of lymphoma.

• Discrepant flow results should make you think twice but should not necessarily change your mind.
FISH: Fluorescence *in situ* Hybridization

- Detection of specific, defined abnormalities
- Relatively rapid turn-around (24-48 hrs)
- Can be performed on fixed, paraffin-embedded tissues

- **Break-apart probes:**
  - *Separation* of the signals is abnormal.

- **Fusion probes:**
  - *Fusion* of probe signals is abnormal.
FISH for t(14;18) IGH/BCL2

IGH/BCL2 fusion probe.
FISH for *MYC* Translocations

*MYC* break-apart probe
# FISH Assays Used for Lymphomas

<table>
<thead>
<tr>
<th>Probe</th>
<th>Type</th>
<th>Detects</th>
<th>Lymphoma Type</th>
</tr>
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<tbody>
<tr>
<td>MYC-IGH</td>
<td>F</td>
<td>t(8;14) MYC-IGH</td>
<td>Burkitt, DLBCL</td>
</tr>
<tr>
<td>LSI-MYC</td>
<td>BAP</td>
<td>8q24 MYC</td>
<td>DLBCL, Burkitt</td>
</tr>
<tr>
<td>BCL6</td>
<td>BAP</td>
<td>3q27 BCL6</td>
<td>DLBCL, Follicular</td>
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<tr>
<td>IGH-BCL2</td>
<td>F</td>
<td>t(14;18) IGH-BCL2</td>
<td>Follicular, DLBCL</td>
</tr>
<tr>
<td>IGH-CCND1</td>
<td>F</td>
<td>t(11;14) Cyclin D1</td>
<td>Mantle Cell</td>
</tr>
<tr>
<td>ALK</td>
<td>BAP</td>
<td>2p23 ALK</td>
<td>ALK+ ALCL</td>
</tr>
<tr>
<td>6p25.3</td>
<td>BAP</td>
<td>DUSP22/IRF4</td>
<td>ALK- ALCL, B-cell</td>
</tr>
<tr>
<td>3q28</td>
<td>BAP</td>
<td>TP63</td>
<td>PTCL, ALK- ALCL</td>
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</table>

F=Fusion, BAP= Breakapart probe
Genetically Defined Lymphomas

• ALK positive ALCL t(2;5) in ~85% of cases.
  » Immunohistochemistry is a reliable surrogate and detects variant translocations as well.

• Large B-cell lymphoma with *IRF4* rearrangement
  » Provisional entity, more later...

• Mantle cell lymphoma t(11;14) in >95% of cases.
  » Immunohistochemistry is a reliable surrogate
    ▪ Will detect rare variant translocations with light chain genes.
  » Cyclin D1 negative cases exist.

• High grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangement
Lymphomas with highly characteristic genetic changes that do not define them:

- Burkitt lymphoma: \( IG-MYC, t(8;14), t(2;8), t(8;22) \)

- Follicular lymphoma: \( IG-BCL2 \ t(14;18) \)

- Lymphoplasmacytic lymphoma: \( MYD88 \) mutation.

- Hairy cell leukemia (\( BRAFV600E \)).
When should I send for clonality?

• Most diagnoses of lymphoma do NOT require molecular testing.

• Useful in difficult cases, usually where the differential includes an atypical reactive process.

• Comparing separate lesions (both spatially and chronologically).

• Will it change your diagnosis?
  » Will you call it lymphoma if positive?
  » Will you NOT call it lymphoma if negative?
Pitfalls of Clonality Testing

• Failed amplification
  » Low quantity, poor quality DNA
    ▪ Small or poorly-fixed specimen

• False negatives
  » Somatic hypermutation (85% sensitivity in follicular lymphoma)
  » Sampling wrong area

• False positives
  » Clonal selection in non-neoplastic processes
  » Limited sampling area

• Determining lineage (T vs. B)
  » Beware of lineage infidelity
    ▪ Much more common in immature neoplasms
Genomic (NGS) Testing in Lymphoma: Role Still Limited

- Lymphoma diagnosis still based on morphology and immunophenotype.
  - Point mutations associate with lymphoma subtypes but do not define them.
- Importance of translocations, which are technically challenging to assess for by NGS.
  - RNA-based assays.
- Likely to increase as targeted therapy becomes a reality.
CLL Mutation Panel by NGS

- 27 gene panel:
  - ATM, BCL2, BIRC3, BRAF, BTG1, BTK, CARD11, CD79B, CXCR4, DDX3X, FBXW7, IKZF3, KRAS, MAP2K1, MED12, MGA, MYD88, NOTCH1, NRAS, PLCG2, POT1, RPS15, SAMHD1, SF3B1, TP53, XP01, ZMYM3

- Useful in conjunction with traditional CLL biomarkers (e.g., IGHV mutation status, 17p deletion)

- Assessment for BTK resistance mutations in CLL patients
  - High frequency in patients who progress on BTK inhibitor therapy (e.g. ibrutinib, acalabrutinib)
    - Mutations in BTK (e.g., C481S) that confer resistance
    - Mutations in PLCG2, CARD11, CD79B, CXCR4, MYD88 implicated in primary or acquired resistance

Slide courtesy of Jay Patel
Is Ancillary Testing Required?

• Immunophenotyping considered essential for most diagnoses.

• It is not always necessary to do all testing to make a definitive diagnosis.
  » Clinical guidelines like the NCCN advocate specific testing, often beyond what is required to diagnose.

• As more information becomes available (i.e., molecular profiling), more extensive testing may be required for some entities.
Diffuse Large B-cell Lymphoma

- Ancillary testing required for diagnosis: CD20.
**Diffuse Large B-cell Lymphoma Ancillary Testing**

- Useful for sub-classification and/or prognostic information
  - EBER
    - *EBV positive DLBCL, NOS*
  - FISH for *MYC, BCL2, BCL6* translocations
    - *High grade B-cell lymphoma with MYC and BCL2+/−BCL6*
  - CD10, BCL6, MUM1
    - *DLBCL NOS, germinal center vs. activated B-cell subtypes*
  - MYC, BCL2 protein co-expression
    - Does not define an entity but has prognostic significance
**MYC and BCL2 Rearrangements and Protein Expression: Inform Prognosis and Guide Therapy**

- Diffuse large B-cell lymphoma, NOS
- Double-expresser (DE) DLBCL, NOS
  - Expresses MYC (>40%) and BCL2 (>50%) protein
  - 30% of DLBCL
- High grade B-cell lymphoma double hit (HGBL-DH), 4-6% of DLBCL.
  - **MYC/BCL2**, 80% (includes 20% triple hit).
  - **MYC/BCL6**, 20%.
MYC and BCL2 Double Hit or Double Expresser: Prognosis

79-year-old woman with recent breast cancer diagnosis and enlarged lymph nodes.

• Right neck mass.
Results of FISH and Final Classification

• MYC breakapart probe assay negative.
  » MYC rearrangement excluded.
  » MYC/BCL2 or BCL6 double hit lymphoma excluded.

• Classification:
  » Diffuse large B-cell lymphoma, activated B-cell subtype, with co-expression of MYC and BCL2.

• Prognosis: bad, but not as bad as MYC/BCL2 double hit.

• Consider DA-EPOCH-R rather than R-CHOP.
Diagnosis of DLBCL requires only morphology and immunophenotype.

Ancillary testing can inform prognosis and identify recognized subtypes.

Diagnosing or excluding the WHO 2017 category HGBL, with MYC+BCL2 +/- BCL6 rearrangement requires FISH.

» A genetically-defined lymphoma.

Testing should be performed when results will affect patient care.
T-cell Lymphomas

• Diagnosis of lymphoma heavily dependent on morphology.
• Immunophenotyping can show aberrancies.
• Molecular or flow cytometry studies can show clonality.
• Neither aberrancies nor clonality is diagnostic of malignancy!
Immunophenotyping in T-cell Lymphomas

- Normal T-cells express CD2, CD3 (surface), CD5, CD7, and CD4 or CD8.
- Aberrancies:
  - Deletion/absence
  - Weaker or stronger than normal
- Relatively soft findings as reactive T-cells can show such aberrancies.
  - Decreased CD7 is common.
- Multiple aberrancies increases suspicion.
- Loss of surface CD3 is highly suspicious.
T-cell Clonality

• Molecular/PCR
  » Detects clonal TCR rearrangements.
  » Fresh or fixed tissue.

• Flow cytometry: V-beta
  » Identifies restricted use of TCR V-beta chains.
  » Allows assessment of an aberrant population.
  » Requires fresh tissue/blood/marrow.

• Both can be positive in reactive T-cell populations!
Be Aware of Non-Neoplastic Clonal T-cells

- There are MANY examples of clonal T-cell proliferations that are NOT neoplastic
  - Common in skin, peripheral blood
  - Post transplant
  - Various immune responses
    - Inflammatory (RA, Crohn’s etc.)
    - Malignancy (CLL/SLL, etc.)
- T-cell repertoire decreases with age so clones more likely.
• 55-year-old man with a reported history of left groin lymphadenopathy,
• Fatigue, night sweats, fever, elevated LDH, mildly elevated AST and ALT.
• Mild lymphocytosis.
Difficult case!

- Diagnosis: EBV-positive T-cell lymphoproliferative disorder (not really a thing).

- What troubled me:
  » Partial effacement, negative flow study, EBV positivity.

- Sent for clonality...came back positive.
  » Remained stubborn.

- Sent to NIH, Dx: Peripheral T-cell lymphoma, NOS with associated EBV-positive B-cell proliferation.
  » Remained stubborn.
Clinical follow up 1 month later

- Patient now totally asymptomatic.
- No abnormal lymphadenopathy.
- EBV titers at biopsy showed high IgM without IgG.
- Now convalescent titers with high IgG as well as IgM.
- Clinically fits best with a primary EBV infection, although unusual at this age.
Considerations for T-cell Lymphoma Diagnosis

• Morphology: is there total effacement by monomorphous atypical cells, or are the atypical cells variable in size and mixed in?

• Ancillary Testing;
  » Definitive phenotypic aberrancies by flow and immunohistochemistry?
  » Strongly consider T-cell clonality.
Case: 9-year-old boy with a mediastinal mass
Immunophenotype and Diagnosis

- Positive for CD2, CD30 (strong and diffuse), TIA1, CD45, CD4 (weak), CD7 (subset).
- Negative: CD20, PAX5, CD15, CD8, CD5.

- Diagnosis: Anaplastic Large Cell Lymphoma (ALCL), ALK1 negative.

- Other considerations:
  - Hodgkin: morphology; CD15-, PAX5-, CD3+
  - PTCL, NOS: CD30 too strong and diffuse.
Additional Studies

- FISH negative for rearrangement of *DUSP22/IRF4*.

- Immunohistochemical stain for p63 is negative in tumor cells.
IRF4/DUSP22 Rearranged ALK- ALCL Shows Outcomes Similar to ALK+ ALCL

Summary: T-cell Lymphomas

• Diagnosis should be based on morphology.
  » Lymph node effaced by atypical cells.
• Immunophenotyping and clonality offer welcome support but beware of “false” positives.
• ALK negative ALCL should get additional prognostic testing.
  » IRF4/DUSP22 FISH
    ▪ Good prognosis if positive
  » TP63 FISH or p63 IHC surrogate
    ▪ Bad prognosis
17-year-old girl with enlarged tonsils

• Original pathology report:
  » R tonsil: malignant lymphoma, favor high grade.
  » L tonsil: follicular hyperplasia.
  » “The overall features favor a high-grade lymphoma.”

• Implications: High-grade implies Burkitt lymphoma in this age group, could include double-hit lymphomas in older adults.

• Intensive chemotherapy regimen required.
Differential Diagnosis

• Burkitt lymphoma: Excluded by morphology
  » Lacks tingible body macrophages, too pleomorphic.
• Follicular lymphoma, grade 3.
• Diffuse large B-cell lymphoma.
• Large B-cell lymphoma with *IRF4* rearrangement.
  » New entity in WHO 2017.
• FISH results:
  » FISH for *MYC*, *BCL6*, and *BCL2* negative.
  » FISH for *IRF4/DUSP22* positive.
Large B-cell lymphoma with IRF4 rearrangement.

- Localized in head and neck.
- Median age 12 (range 4-79).
- Morphologically fit into DLBCL, follicular lymphoma grade 3, or pediatric type follicular lymphoma.
- Positive for BCL6 and IRF4/MUM1.
- Good outcome after chemotherapy.
  - Less intensive therapy than Burkitt lymphoma.
- In the appropriate clinical context, FISH for IRF4/DUSP22 should be performed.
Overall uncommon (<1%), but more common in younger patients

- We studied 32 patients from Children’s Oncology Group protocols.
- FISH for *IRF4/DUSP22* positive in 2/32 cases (6%).
  - One in tonsils.
  - One in ileum.

Summary

• Ancillary testing contributes greatly to lymph node diagnosis.
  » Flow/IHC: in almost every case.
  » FISH: specific applications.
  » Clonality: important in some cases but be aware of false positives and negatives.
  » Genomics: largely still in the future.

• Important to keep the limitations in mind.
  » Stick to your morphologic impression!
Small Needle Core Biopsies

• Small biopsies and cytology lose many morphologic clues.
• Architecture critical to our classification system.
  » Aspirations only: difficult to say much.
  » In lymphoma diagnosis, FNA means “For No Answer.”
• Needle cores at least offer a glimpse of architecture, but the tiny snapshot can be misleading.
Follicular vs. Diffuse
What are you **NOT** seeing?
How specific should I be?

• CD10 positive B-cell lymphoma (flow cytometry was positive).

• This is probably a DLBCL.

• Consider other possibilities:
  » Grade 3B follicular lymphoma
  » Follicle of florid follicular hyperplasia with a small B-cell clone by flow cytometry?
How specific should I be?

- Follicular lymphoma?
- Consider other possibilities:
  - Missing areas of transformation not sampled.
  - BCL2+ B-cells are normal in mantle zones/primary follicles.
    - Confirm BCL6 co-expression.
  - Follicular neoplasia in situ (formerly FL in situ).
- Avoid formal grading.
  - “No high grade seen.”
Problematic Diagnoses in Cores

Large B-cell Lymphoma with IRF4 rearrangement.

EBV lymphadenitis
Problematic Diagnoses in Cores

• Nodular lymphocyte predominant Hodgkin lymphoma vs. T-cell histiocyte rich large B-cell lymphoma.
  » NLPHL can have extensive diffuse areas that look just like TCHR.

• Hodgkin lymphoma with few HRS cells.
  » Ok in right clinical setting with multiple cells, typical immunophenotype.
  » Beware if primary mediastinal or EBV driven LPD is in the differential.

• Lymphoma vs. in situ neoplasia (FL, MCL).

• T-cell lymphoma—architecture assessment from low power is critical.
Summary

- Needle core biopsies present a significant challenge in lymphoma diagnosis.
- Cannot assess architecture.
  - Critical in classification system.
  - Limits ability to assign precise diagnosis.
- Limited sampling may not represent entire node.
  - Ask yourself: What am I not seeing?
- Use caution and consider a less specific diagnosis, e.g. “B-cell lymphoma.”
- More and more common due to less invasive nature.
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