Molecular Tools in the Diagnosis of Lymphoma

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Learning Objectives

• To be familiar with common algorithms that incorporate FISH testing in the work up of diffuse large B-cell lymphoma

• To understand when it is appropriate to use molecular clonality testing in the work up and diagnosis of lymphoma

• To be familiar with the limitations and “pitfalls” of clonality testing
Modifications to DLBCL category

- DLBCL
  - Germinal center B-cell type
  - Activated B-cell type
- TCHRLBCL
- Primary CNS
- Primary cutaneous DLBCL, leg type
- EBV+ DLBCL, NOS
- EBV+ mucocutaneous ulcer
- Intravascular LBCL

- High grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements
- High grade B-cell lymphoma, NOS
- ALK+ large B-cell lymphoma
- HHV8+ DLBCL, NOS
- Large B-cell lymphoma with IRF4 rearrangement

Diffuse Large B-cell Lymphoma

- How do you work this up?
- What is sufficient?

Diffuse Large B-cell Lymphoma Ancillary Testing

- Ancillary testing for sub-classification and/or prognostic information
  - GC vs. non-GC subtyping
  - FISH for MYC, BCL2, BCL6
  - Immunohistochemistry for MYC, BCL2
  - ISH for EBV (EBER)
Microarray analysis identified two distinct gene expression patterns in DLBCL

- Germinal center B-cell (GC) group
- Activated B-cell (ABC) group
- 50-60% of adult DLBCL are GC

Alizadeh et al., Nature 403:503, 2000

GC gene expression profiles were associated with a better overall survival, independent of IPI

Alizadeh et al., Nature 403:503, 2000
IHC Subtypes of DLBCL

CD10
+ GC
- ABC

BCL6
- MUM1
+ ABC

Hans et al., Blood, 2004

2011: R-CHOP Era
Outcome of DLBCL according to molecular subtype (GCB vs ABC).

Progression-free survival

Overall survival

Patients treated with R-CHOP (n=52)

Outcome of 157 DLBCL patients according to GCB vs non-GCB profile as assessed by 5 immunohistochemistry algorithms.

IHC Algorithms:
A: Colomo
B: Hans
C: Muris
D: Choi
E: Tally

Patients treated with R-CHOP
Cell of Origin Subtyping in DLBCL

- Difference in prognosis is smaller in patients treated with R-CHOP than CHOP.
- Gene expression profiling can still segregate these groups.
- Immunophenotyping approaches cannot reliably separate groups with distinct prognoses.
- Testing may have emerging role for guiding targeted therapy.

MYC and BCL2 Rearrangements and Protein Expression: Inform Prognosis and Guide Therapy

- Diffuse large B-cell lymphoma, NOS
- Double-expressor (DE) DLBCL, NOS
  - Expresses MYC (>40%) and BCL2 (>50%) protein
  - Poor prognosis
- High grade B-cell lymphoma double hit (HGBL-DH), 4-6% of DLBCL.
  - MYC/BCL2, 80% (includes 20% triple hit).
  - MYC/BCL6, 20%.

Prognostic impact of MYC/BCL2 coexpression in DLBCL. (A-B) OS (A) and PFS (B) of patients with DLBCL with MYC/BCL2 coexpression (MYC+BCL2+) in the training set.

Prognostic impact of MYC/BCL2 coexpression in DLBCL risk-stratified according to clinicopathologic parameters.


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MYC/BCL2 coexpression contributes to the inferior prognosis of ABC-DLBCL.


©2013 by American Society of Hematology

Prognostic impact of MYC/BCL2 coexpression in DLBCL is independent of MYC/BCL2 corearrangement and TP53 mutation status.


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Key Points from Hu et al.

- MYC/BCL2 protein co-expression is found in ~30% of de novo DLBCL.
- These patients have a poor clinical outcome with a 5-year OS and PFS of <30%.
- MYC/BCL2 co-expression correlates with ABC subtype, so the latter is NOT an independent negative prognostic factor.
- MYC/BCL2 co-expression is a negative prognostic factor independent of MYC/BLC2 double hit.
- MYC/BCL2 co-rearranged (double hit) DLBCLs are rare (10/394 cases); 8/10 had MYC/BCL2 protein co-expression.

MYC/BCL2 Co-Expression Contributes to Inferior Prognosis of ABC subtype

- Presence of MYC/BCL2 co-expression was significantly correlated with the ABC subtype.
- After excluding patients with MYC/BCL2 co-expression, the prognosis of patients with ABC subtype was similar to that of GCB subtype.

“Double Hit” Lymphoma

- Have two of these three genetic abnormalities
  - MYC
  - BCL2
  - BCL6
- Morphology may appear to be DLBCL or may have features that overlap with Burkitt lymphoma
- Aggressive clinical behavior—may require different therapy than DLBCL.
High-Grade B-cell Lymphoma with MYC and BCL2 and/or BCL6 Rearrangements (WHO 2016)

- Aggressive presentation, often disseminated (PB, BM, CSF).
- Can resemble BL with increased pleomorphism and/or atypical immunophenotype or genetic features.
- MYC complex karyotype is common.

MYC/BCL2 Double Hit Lymphomas Have a Poor Prognosis

Hu et al. Blood 2013;121:4021-4031

**Prognostic Impact of Single Hits**

Entire cohort

GCB Subtype

ABC Subtype

Ye Oncotarget 2015
Prognostic Impact of Double Hits

Only MYC/BCL2 Pts. Show Worse Survival

Re-thinking Double Hits

• MYC/BCL6 DHLs do not have a worse prognosis and should not be grouped with or treated as MYC/BCL2 DHLs.
• MYC/BCL6 DHLs do not have a different gene expression profile.
  – BCL6 partners and expression levels vary.
  – 36% of MYC/BCL6 have low MYC expression.
Incidence of Double Hits

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYC</td>
<td>11.8</td>
</tr>
<tr>
<td>BCL2</td>
<td>13.6</td>
</tr>
<tr>
<td>BCL6</td>
<td>23.1</td>
</tr>
<tr>
<td>MYC / BCL2</td>
<td>2.8</td>
</tr>
<tr>
<td>MYC / BCL6</td>
<td>2.0</td>
</tr>
<tr>
<td>BCL2 / BCL6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

- MYC and BCL2 more common in GCB.
- BCL6 more common in ABC.
- MYC/BCL2 almost all in GCB (19/20).
- MYC/BCL6 in GCB and ABC.


DLBCL Prognostic Testing Strategy

De novo DLBCL (excludes transformation, relapse, PTLD unless specifically requested by clinician)

- **Clinical and/or morphology suggests DLBCL subtype:**
  - TCHRBCL
  - EBV+ DLBCL of elderly
  - Primary mediastinal (PMBL)
  - Primary CNS
  - Primary cutaneous leg type

- **MYC and BCL2 Immunohistochemistry:** CD10, BCL6, and MUM1 for Hans COO; MYC FISH

  If pos., BCL2, BCL6

- **FISH:**
  - Fluorescence in situ Hybridization
    - Detection of specific, defined abnormalities
    - Relatively rapid turn-around (24-48 hrs)
    - May be performed on fresh or paraffin-embedded tissues

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

  - Fusion probes:
    - Fusion of probe signals is abnormal.

FISH: Fluorescence in situ Hybridization

Testing not indicated
**FISH for t(14;18) IGH/BCL2**

IGH/BCL2 fusion probe.

**FISH for MYC Translocations**

MYC break-apart probe

**DLBCL Prognostic Testing Strategy**

De novo DLBCL (excludes relapse, PTLD, transformation?)

Clinical and/or morphology suggests DLBCL subtype:
- TCHRBCL
- EBV+ DLBCL of elderly
- Primary mediastinal (PMBL)
- Primary CNS
- Primary cutaneous leg type

Testing not indicated
Challenge: Data Do Not Support the Current WHO Definitions

- **MYC/BCL6** DHLs do not have a worse prognosis and should not be grouped with or treated as **MYC/BCL2** DHLs.
- **MYC/BCL6** DHLs do not have a different gene expression profile.
  - **BCL6** partners and expression levels vary.
  - 36% of **MYC/BCL6** have low MYC expression.

DLBCL Conclusions

- Diagnosis of DLBCL requires only morphology and immunophenotype.
- Diagnosing or excluding the WHO 2016 category HGBL, with MYC+BCL2 +/- BCL6 rearrangement requires FISH.
- Best approach is evolving and lacks consensus at this time.
- Testing should be performed when results will affect patient care.

Clonality Testing
Humoral immunity. Naive B cells recognize antigens and are activated to proliferate and to differentiate into antibody-secreting plasma cells. Some of the activated B cells undergo heavy-chain class switching and affinity maturation, and some become long-lived memory cells. Antibodies of different heavy-chain classes (isotypes) perform different effector functions, shown on the right. Note that the antibodies shown are IgG, these and IgM activate complement, and the specialized functions of IgA (mucosal immunity) and IgE (mast cell and eosinophil activation) are not shown.

Receptor diversity

- 100 million to 1 billion different receptor specificities in one individual
- Diversity is generated by
  - Different segments
  - Different combinations of segments
  - Junctional diversity during recombination
  - Somatic hypermutation
Figure 1. Immunoglobulin heavy chain gene rearrangement. Most PCR tests for this rearrangement use consensus primers directed against the framework three (FR3) region and the heavy chain joining (CH1 or FRIV) region of the rearranged product.

Arber, JMO 2000
Assumption of Clonality in Cancer is Critical to Diagnostic Tools (Flow, Molecular)

Lymphoma Diagnosis

- Morphology
- Immunohistochemistry
- Flow cytometry
  - This is enough! (Most of the time...)

https://doi.org/10.3389/fsurg.2016.00021
How should this test be used?

• Many/most diagnoses of lymphoma do NOT require molecular testing
  – Morphology and immunophenotype are sufficient
• Useful in difficult cases; usually where the differential diagnosis is an atypical reactive process
• Determining lineage (T vs. B)
  – Lineage infidelity
    • Much more common in immature neoplasms
• Comparing separate lesions (both spatially and chronologically)

MALT lymphoma

• Marginal zone (Mucosa associated lymphoid tissue) lymphoma
  – Low grade B-cell lymphoma
  – Some relationship to underlying chronic inflammation
  – Often in extranodal locations
    • Gastrointestinal (usually stomach)
    • Parotid gland, salivary glands, thyroid
    • Eye, lacrimal glands
    • Lung
    • Skin

A&C – at presentation
B&D – after treatment

Gastric biopsies are usually small
Extent of infiltration?

B-cell Clonality Assay:

Pitfalls of Clonality Testing

- Failed amplification
  - Low quantity
  - Poor quality (FFPE)
- Sampling
  - Pseudoclones
  - Wrong area
- False negatives
  - Somatic hypermutation (Follicular lymphoma)
  - Sampling wrong area
  - Clone too small; high reactive background
- "False positives"
  - Clonal proliferation in non-neoplastic processes
Clonal expansion as part of normal immune response


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\[\text{Sampling...}\]
Pitfalls of Clonality Testing

- Failed amplification
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  - Clonal selection in non-neoplastic processes
When to use T-cell clonality testing?

- There are MANY examples of clonal T-cell proliferations that are NOT neoplastic
  - Commonly skin, peripheral blood
  - Post transplant
  - Various immune responses
    - Inflammatory (Crohn’s etc.)
    - Malignancy (CLL/SLL, etc.)
- Still can be very helpful in tissues (lymph node, etc.) that look like a T-cell lymphoma, but more evidence/support is needed.
T-cell receptor rearrangement

- TRD -> TRG -> TRB -> TRA
- This happens in all T-cells, regardless of αβ or γδ expression
- Thus, all αβ T-cells (the most common subset) will have identifiable (but not expressed) TRG rearrangements

*Figure 6.* The T cell receptor γ chain locus on chromosome region 7p15 contains a limited number of variable and joining region genes that make it ideal for PCR amplification of the rearrangements.

*Arber, JMD 2000*
Mycosis Fungoides – a common T-cell lymphoma of the skin

Mycosis fungoides

Pathology outlines.com

Staging of mycosis fungoides and Sezary syndrome often involves evaluation of the peripheral blood for tumor cells, which may include TCR molecular studies if tumor cells are suspected by morphology.
Non-Neoplastic Clonal T-cells

- There are MANY examples of clonal T-cell proliferations that are NOT neoplastic
  - Commonly skin, peripheral blood
  - Post transplant
  - Various immune responses
    - Inflammatory (Crohn's etc.)
    - Malignancy (CLL/SLL, etc.)
Example from ESRD patients – Peripheral blood T-cells

T-cell repertoire decreases with age

The future...

- Using NGS data for T-cell clonality
  - More powerful
  - Not just used for clonality, but can examine different types of T-cell immune responses in other non-hematologic malignancies
  - May alter therapy choices; immune checkpoint inhibitors

- The downside
  - Longer TAT
  - Higher cost
  - Clones may be readily identified and still does not solve the problem that clonality ≠ lymphoma!

NGS in recurrence

Identification of Clonal TCR Sequence in Initial Time Point

Subsequent Biopsy Time Points

Determine if initially identified clonal sequence is still present

Detect aberrant clonal expansions

Determine if variants look similar

Fig. A: Mycosis fungoides. A. Representative skin biopsy specimen characterized by a lymphocytic infiltrate composed of small to medium atypical lymphocytes demonstrating epidermotropism. Helper cells are notable within the epidermis. Hematoxylin and eosin stain, original magnification ×200. B. Lymphocytes were immunoreactive for CD2, CD3, and CD5, with reduced CD7 positivity. As CD4 also stains Langhans cells in the epidermis, there are more CD3+ cells than CD4+ cells in the epidermis. Because of this, it is important to compare CD3 and CD8 when examining the epidermal compartment. The CD8+/CD4+ cells likely correspond to CD8+ T cells. CD4 expression was greater than that of CD8. (Immunohistochemistry, original magnification ×20.)
Conclusions

- DLBCL work-up is constantly evolving but IHC and FISH are important for prognosis

- Molecular clonality assays can be very helpful if used in the right context, with an awareness of possible “pitfalls”.
  - Most importantly they should be combined with impression from all other studies and history