The Role of Molecular Diagnostics in Characterizing Cutaneous Lymphoproliferative Disorders

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September 27, 2019
Outline

- T cell clonality and usefulness in early mycosis fungoides or Sézary syndrome
  - Dual TCR PCR using the gamma gene locus
  - The usefulness of studying TCR beta separately or in conjunction with TCR gamma
Outline

• B cell clonality and usefulness in low grade B cell lymphomas
  – IgH and IgK in tandem
  – Multiple Biopsies
Introduction

• The diagnosis of cutaneous lymphoma is not always straightforward, as there can be significant overlap with reactive entities
• Careful correlation with clinical features is essential for an accurate diagnosis
Introduction

• In the context of mycosis fungoides and Sézary syndrome, both immunohistochemical approaches and molecular studies can be used as ancillary diagnostic methods.

• Especially useful if histologic findings are ambiguous (Comfere 2018; Vidal 2018)
Introduction

• Immunohistochemical studies can lack usefulness if:
  – The infiltrate overall is scant
  – A large reactive component is present which masks the neoplastic cells

• Large studies have shown false negative rates of 23% (Smoller 1995)
Molecular Diagnostics

• Much attention has been focused on the role of PCR or Southern blot analysis for the detection of clones.

• Malignant lymphocytic processes are characterized by the presence of a single clone, whereas reactive infiltrates are polyclonal.
Molecular Diagnostics

- Southern blot analysis initially thought to be gold standard
  - Cannot be performed on paraffin tissues
  - Sensitivity of this analysis is low especially in context of high reactive background (i.e., early MF)

- Replaced by PCR analysis
Molecular Studies-A Word about High Throughput Sequencing

- Complete sequencing of the CDR3 region (encodes TCR beta and TCR gamma)
- Quantify number of clones, proportion of specific clones, and sequences of clones
- More sensitive and specific than current PCR methods (Sufficool 2015)
Molecular Studies-A Word about High Throughput Sequencing

• May have greater predictive abilities for unfavorable clinical outcomes in early disease
  – Tumor clone frequency may be predictive of aggressive later disease (Kirsch 2015, de Masson 2018)

• Expensive (Fujii, 2019)
Molecular Diagnostics

• Reactive conditions have had numerous documentations of clonality
  – Pityriasis lichenoides et varioliformis acuta and pigmented purpuric dermatitis are two well documented conditions (Dereure O 2000; Crowson 1999)
Problems with Use of PCR as Ancillary Test

• No standardized methodology existed prior to BIOMED-2
  – PCR analysis at different institutions could not be directly compared

• Positive and negative studies among neoplastic and reactive conditions had not been directly compared and correlated with clinical outcome

• Positive results for reactive conditions in T cell clonality analysis were high (25-64%, Thurber 2007)
Molecular Diagnostics

• A standardized series of primers and protocols was developed in Europe for ease of inter-laboratory comparisons (BIOMED-2 collaborative study)

• The TCR gamma (TCRG) gene locus is most often targeted
Molecular Diagnostics

- Using new standardized protocols, a high rate of rearrangements was reported in T cell malignancies in general (89% TCRG, 94% TCRB, van Dongen 2003)
Initial Studies-dual TCR PCR

• To maintain the high level of sensitivity in this analysis, and increase the specificity, we elected to examine analysis of TCRG gene rearrangements via dual PCR

• This entailed examination of two or more biopsies of suspicious skin lesions via PCR
  – Sequential lesions over time or multiple biopsies performed at the same time
Dual TCR PCR (Thurber 2007)

• 46 patients were studied overall (retrospective study)
• 10 patients had unequivocal diagnoses of MF on clinical and histologic grounds
• 18 patients had inflammatory conditions (i.e., psoriasis, eczema, pigmented purpuric dermatitis, arthropod bite reaction, etc.)
Dual TCR PCR

• 18 patients had lesions that could not be initially classified (indeterminate), but were found to be either MF or inflammatory dermatoses (ID) after follow up of 12-136 months
Dual TCR PCR

- 5 mm punch biopsies were employed, and most cases were of patients who had had at least 2 biopsies performed at the same time.
- Using established BIOMED-2 protocols and primers for TCRG, virtually all V-J combinations can be assessed.
Dual TCR PCR

• Gene scan analysis allows PCR products of different sizes to be compared
## Results

<table>
<thead>
<tr>
<th>Identical TCRG</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unequivocal MF</td>
<td>8/10 (80%)</td>
<td></td>
</tr>
<tr>
<td>Indeterminate-MF</td>
<td>11/13 (84.6%)</td>
<td>19/23 (82.6%)</td>
</tr>
<tr>
<td>Unequivocal ID</td>
<td>0/18 (0%)</td>
<td></td>
</tr>
<tr>
<td>Indeterminate-ID</td>
<td>1/5 (20%)</td>
<td>22/23 (95.7%)</td>
</tr>
</tbody>
</table>
Dual TCR PCR

• In 2/18 patients with unequivocal inflammatory dermatoses, one biopsy yielded a clone; however, no patients with unequivocal inflammatory dermatoses had identical clones.

• The lack of an identical clone can be a powerful indicator of a reactive process (specificity 95.7%).
Limitations of Initial Study

• Sensitivity of test regarding early MF lesions was not as high as one would want, even with dual TCR PCR (82.6%)

• Number of both types of lesions, but especially inflammatory dermatoses studied, is small
Role Of TCRB

- TCRB primers and protocols available
  - Not used as often
  - Greater number of recombinations
  - Greater chance that primer combinations may not cover all possibilities
TCRB

- In initial BIOMED-2 protocol studies, TCRG rearrangement rate=89% and TCRB rearrangement rate=94%\(^4\)
- Also reported that addition of TCRB study increased clonality detection to 94% when fresh/frozen samples are used (Bruggemann 2007)
TCRG/TCRB Comparisons

• Interested in understanding if addition of TCRB to a negative TCRG study could increase sensitivity in early MF (Zhang 2010)
TCRB Study in MF/ID

- We studied 69 samples from MF patients and 133 samples from patients with inflammatory dermatoses (ID) (Zhang 2010)
- In most cases of MF TCRG was performed first (usually as part of routine testing) and TCRB was added as part of the study
- Follow up available for 80 patients (median 30.5 months)
# TCRG/TCRB Results

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCRG</strong></td>
<td>63.8% (44/69)</td>
<td>36.2% (25/69)</td>
<td>16.5% (22/133)</td>
<td>83.5% (111/133)</td>
</tr>
<tr>
<td><strong>TCRB</strong></td>
<td>63.8% (44/69)</td>
<td>36.2% (25/69)</td>
<td>16.5% (22/133)</td>
<td>83.5% (111/133)</td>
</tr>
<tr>
<td><strong>TCRG and TCRB</strong> *&lt;br/&gt;(with concordant results)</td>
<td>49.3% (34/69)</td>
<td>50.7% (35/69)</td>
<td>6.8% (9/133)</td>
<td>93.2% (124/133)</td>
</tr>
<tr>
<td><strong>TCRG or/and TCRB</strong> <strong>&lt;br/&gt;(at least one)</strong></td>
<td>78.3% (54/69)</td>
<td>21.7% (15/69)</td>
<td>26.3% (35/133)</td>
<td>73.7% (98/133)</td>
</tr>
</tbody>
</table>
## TCRG/TCRB Results

<table>
<thead>
<tr>
<th></th>
<th>TCRG (positive/total)</th>
<th>TCRB (positive/total)</th>
<th>TCRG and TCRB (positive/total)</th>
<th>TCRG or TCRB (positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>3/10 (30%)</td>
<td>6/10 (60%)</td>
<td>2/10 (20%)</td>
<td>7/10 (70%)</td>
</tr>
<tr>
<td>T2</td>
<td>10/11 (90.9%)</td>
<td>7/11 (63.6%)</td>
<td>7/11 (63.6%)</td>
<td>10/11 (90.9%)</td>
</tr>
<tr>
<td>T3</td>
<td>11/15 (73.3%)</td>
<td>9/15 (60%)</td>
<td>9/15 (60%)</td>
<td>11/15 (73.3%)</td>
</tr>
<tr>
<td>T4</td>
<td>6/8 (75%)</td>
<td>6/8 (75%)</td>
<td>6/8 (75%)</td>
<td>6/8 (75%)</td>
</tr>
</tbody>
</table>
Summary of Results

• When used alone, sensitivity and specificity for TCRG and TCRB are nearly identical
• If both tests have to be positive for a positive result, specificity is maximized (93%) at the expense of sensitivity (49%)
Summary of Results

• If either test can be positive for a positive result, sensitivity is increased to 78% but specificity drops to 73%
Results as a Factor of T stage

• Adding TCRB to TCRG is helpful in confirming MF at stage T1, but does not augment the diagnosis at other stages (T2-4)
Proposed Algorithm for Use of TCRB in a Clinical Context

• Pre-test probability measures how likely it is that the patient has MF before the clonality assay is run

• Addition of TCRB not useful if pre test probability is very low (obviously NOT MF) or very high (obviously MF)
Proposed Algorithm for Use of TCRB in a Clinical Context

• If pre-test probability is moderate to high, and TCRG is run first and is negative, a positive TCRB can be confirmatory of MF

• If pre-test probability is low to moderate, and TCRG is run first and is positive, a positive TCRB can be confirmatory of MF

• Maximizes the role of TCRB in a clinical context
Case 1

- 43 year old man with 8 year history of asymptomatic red patches on BLE, arms and trunk
- The clinical differential diagnosis includes nummular eczema, MF, irritant dermatitis
- On examination, small coin-shaped lesions, primarily on the upper thighs
- Clinical suspicion for MF is low
Case 1

- Additional clinical history revealed that the patient had been using topical steroids
- This was stopped and the patient rebiopsied two weeks later
BIOPSY 2
Biopsy on topical therapy (biopsy 1)

Biopsy off topical therapy (biopsy 2)

Biopsy off topical therapy (biopsy 3)

Single peak in V9 in same location in all three biopsies
Case 2

- Male with rash, suspicious clinically for mycosis fungoides
- Two biopsies are taken
Additional History

• The patient was newly diagnosed with HIV
• The rash was evaluated by our cutaneous oncology expert and deemed clinically to not be compatible with mycosis fungoides
Negative clonality assay
Case 3

• Male with rash on arm, suspicious clinically for mycosis fungoides
Biopsies 1 and 2 each have a single identical clone in the V9 region.

The patient had three more biopsies with identical histologic features and identical clonality results.
Granulomatous MF

• Rare but well defined variant of MF, thought to be a granulomatous infiltrate obscuring the otherwise defined features of MF
• Can be difficult to diagnose on morphology alone
• Dual TCR PCR can be very helpful in confirming the diagnosis
Cutaneous B Cell Lymphoma

- Rarer than T cell lymphomas (MF)
- Can be difficult to distinguish low grade B cell lymphomas (follicle center lymphoma and marginal zone lymphoma) from pseudolymphoma
Cutaneous B cell Lymphoma

• Immunohistochemistry
  – Cutaneous FCL differ from nodal FL in that bcl-2 and CD10 are often not expressed
  – In MZL, light chain restriction is often difficult to demonstrate via in situ hybridization/immunohistochemistry on paraffin embedded tissues
Molecular Diagnostics

• PCR analysis targets the V and J constant regions of the immunoglobulin heavy chain

• Lack of standardization and inability to compare protocols limit usefulness of this approach (range of positivity in literature varied from 34% to 85% in lymphomas) (Morales 2008)
BIOMED-2 Protocols

• Designed to have wide analytical approach to IgH rearrangements
• Had separate protocol to cover IgK and IgL rearrangements
• Also designed primers to detect t(14;18)
• 41 primers developed overall
BIOMED-2 Protocols

• Highly sensitive and specific for B cell malignancies

• Concentrated primarily on nodal lymphomas, and primarily on frozen/fresh tissues
BIOMED-2 Protocols to test Cutaneous BCL

• We elected to examine the ability of these protocols to detect FCL and MZL using well established patients with lymphoma and FFPE tissues (Morales 2008)

• IgH, IgK and IgKde (immunoglobulin kappa deleting element) were targeted
The Initial Study

• 26 patients overall with lymphoma, 15 with MZL, 11 with FCL
  – Well characterized patients with follow up times from 24-64 months
• 23 patients with pseudolymphoma were selected
## The Initial Study

<table>
<thead>
<tr>
<th></th>
<th>Cases positive for clone (Combined IGH and IGK) (%)</th>
<th>Cases positive for IGH alone (%)</th>
<th>Cases positive for IGK alone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBCL</td>
<td>22/26 (85)</td>
<td>18/26 (69)</td>
<td>17/26 (65)</td>
</tr>
<tr>
<td>MZL</td>
<td>12/15 (80)</td>
<td>11/15 (73)</td>
<td>9/15 (60)</td>
</tr>
<tr>
<td>FCL</td>
<td>10/11 (91)</td>
<td>7/11 (64)</td>
<td>8/11 (73)</td>
</tr>
<tr>
<td>Benign lymphoid infiltrates</td>
<td>1/23 (4)</td>
<td>1/23 (4)</td>
<td>None</td>
</tr>
</tbody>
</table>
Initial Study

• The BIOMED-2 protocol allowed for high sensitivity (85%) and specificity (96%) in this context
• Higher than previously reported detection rate of 56% (Lukowsky 2006)
• Subsequent reports showed similar findings (Felcht 2011)
Addition of IgK to IgH analysis

- Addition increased clonality detection rate from 69% to 85% in biopsies of CBCL
- In FCL patients, clonality detection with IgK (73%) is higher than with IgH (65%)
- May be due to lack of somatic hypermutation in IgK gene locus
The Role of Testing Multiple Biopsies

• We next sought to examine whether multiple biopsies were helpful in cementing the diagnosis of lymphoma in CBCL (Fujiwara 2012)
Multiple Biopsies

• 20 patients with CBCL were studied who had two or more biopsies
• 16 had two or more sites biopsied, either at the same time point or at different time points
• 4 had the same lesion biopsied at different time points
Multiple Biopsies

• These were compared to 12 patients with benign infiltrates who had more than one biopsy performed
• Both IgH and IgK clonality assays were performed
• Sequencing was performed in positive cases
Multiple Biopsies

• In some cases with reduced amplification in one of the paired samples, patient specific primers were generated from a positive clonality study to look for clones in the other sample
Results

• Positive clone found in 19/20 patients with CBCL (all samples are counted)
  – 11/12 patients with MZL (92%); 8/8 patients with FCL (100%)
  – 2/12 patients with benign infiltrates also had positive clones (17%)
Results

• Identical clones found in 11/20 patients with CBCL (55%) (8 MZL, 3 FCL)

• No identical clones were found in patients with BLI (0/12)
Results

• When all data points are counted, sensitivity increases from 85% (last study) to 95% (this study)
• Specificity decreased from 96% (last study) to 83% (this study)
• Both patients with benign infiltrates who had clones identified had lupus
  – One showed no evidence of lymphoma upon clinical follow up
Results

• With identical clone identification, sensitivity dropped from 95% to 55% but specificity increased from 83% to 100%
• Results significantly affected by small numbers of samples
Practical Applications

• Dual clonality assays, in this context, somewhat increase specificity but significantly decrease sensitivity
• May be useful in the context of the rare reactive process that may show a clone
  – If repeat studies over time show negative clonality studies, may be more confident of a reactive process
Case 4

• 75 year old male with lesions on trunk suspicious for cutaneous lymphoma
Kappa ISH
Lambda ISH
Identical clone found in 2 different lesions
Summary

• It continues to be a challenge to distinguish between early lesions of mycosis fungoides and reactive infiltrates
• Dual TCR PCR provides excellent sensitivity and specificity
• High throughput sequencing is much better, but very expensive
Summary

• When is addition of TCRB assessment to TCRG assessment on single biopsies useful?
  – Add TCRB to a negative TCRG if MF is suspected
  – Add TCRB to a positive TCRG if MF is not suspected
Summary

• Low grade B cell lymphomas of the skin are difficult to diagnose as overlap with reactive infiltrates is relatively common
• IgH and IgK clonality assays can be extremely useful, especially in tandem
• Dual positivity in IgH or IgK is indicative of lymphoma
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