International Guidelines for the Diagnosis and Follow up of Sezary Syndrome/Mycosis Fungoides in Peripheral Blood

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Sezary Syndrome / mycosis fungoides group

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  - Rick Sere
  - Susan Richardson
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  - Kristy Wotnekr
  - Others

- Clinicians
  - Michael Girardi
  - Joan Guitart
  - Aaron Mangold
  - Julie Shainbladk
  - Others

Sezary syndrome and Mycosis fungoides

Mycosis fungoides
- Patches and plaques
- Can eventually progress to involve lymph nodes and peripheral blood.

Sezary syndrome
- Erythroderma and blood involvement at presentation.
- Demonstration of peripheral blood involvement is essential for diagnosis.

Role of blood tumor burden in the prognosis of MF/SS
2018 EORTC staging

<table>
<thead>
<tr>
<th>Stage</th>
<th>A1</th>
<th>A2</th>
<th>B1</th>
<th>B2</th>
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<tbody>
<tr>
<td>I</td>
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<td>III</td>
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<td>IIB</td>
</tr>
<tr>
<td>IV</td>
<td>IVA1</td>
<td>IVA2</td>
<td>IVB</td>
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Role of absolute Sezary cell counts in the follow up of patients with MF/SS
2018 EORTC recommendations

- Complete response (CR)
  - B2 (Stage IV) → B0
- Partial response (PR)
  - B2 (Stage IV) → ↓ ≥50%
- Progressive disease (PD)
  - B0/B1 → B2 (Stage IV) AND ↑ ≥50%
- Relapse
  - CR → ↑ ≥1000 cells/µL
- Progressive disease (PD)
  - PR → ↑ ≥1000 cells/µL AND ↑ ≥50%

- Progressive disease (PD)
  - B2 (Stage IV) → ↑ ≥50%
  - B0/B1 → B2 (Stage IV) AND ↑ ≥50%

Adapted from Scarisbrick JJ et al. Eur J Cancer 2018;93:47
Flow cytometric monitoring of Sezary cells to assess response to therapy

Clinical Perspective on Sezary cell testing by flow cytometry: Frustration amongst dermatologists and oncologists

- Hard to tell if an abnormal T-cell population was actually detected.
- Absolute counts are often not reported.
- The phenotype of the abnormal population is often not completely documented.
- The report includes an array of numbers and percentages which are hard to interpret.
- Lack of uniformity and consistency.
- Some clinicians are interested in accessing the flow cytometry histograms to render their own interpretation.

Flow cytometric quantitation of Sezary cells

- EORTC 2018 recommendation:
  - We propose that this [blood involvement by MF/SS] is determined using absolute flow counts of CD4+CD7- or CD4+CD26-.

- Current flow cytometry practice:
  - Comprehensive analysis of T-cell antigen expression (not only CD7 and CD26).
  - Different than normal approach.
  - Gating based on clusters/populations with homogenous phenotypic properties.

Case example: Reactive CD4 T-cells

- CD4 T-cells very commonly show reactive subsets with loss of CD7 and CD26.

Case example: Sezary syndrome with "atypical" phenotype

- Cases of Sezary syndrome or mycosis fungoides with preserved expression of CD7 and/or CD26 are not rare.

Classical and non-classical immunophenotypes of Sezary cells
Case example: Low level involvement by Sézary cells.

- In the setting of low level involvement, CD7+/CD26- Sézary cells largely overlap benign/reactive CD4+ T-cell subsets.

Exclusive assessment of CD7(-) and CD26(-) on CD4+ T-cells is a suboptimal approach to detect and quantify Sézary cells.

Early manuscripts documenting loss of CD7 and CD26 on Sézary cells.

- Other immunophenotypic abnormalities besides CD7 and CD26 are fairly common.

Phenotypic abnormalities of Sézary cells

- 79 blood specimens from 52 patients with MF/SS.
- 27 patients with no hematologic malignancy.
- Dotted lines: Approximate threshold where abnormality is visually evident.

Antibody panel design
Antibody panel design

Basic principles

- Single tube panel.
  - No single antigen can by itself accurately identify Sézary cells.
  - Diagnostic utility of each antigen is highly dependent on its combination with other useful antibodies in a single analysis tube.
  - 6 to 8-color flow cytometry is now broadly available and accessible.
- Selection based on the reported utility of different antigens to discriminate Sézary cells from reactive/benign CD4+ T-cells.
- Review of the literature and experience from contributors.
- Favor basic T-cell antigens.
  - Assay that can be adopted widely (not just in specialty labs).
- Additional markers of potential diagnostic utility

<table>
<thead>
<tr>
<th>Marker</th>
<th>Normal expression</th>
<th>Sézary cells</th>
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<tbody>
<tr>
<td>CD3</td>
<td>Bright positivity on T-cells. Positively reactive in reactive subsets.</td>
<td>Positive. Slight dim expression in 10-50% of cases. Rare partial or complete negativity.</td>
</tr>
<tr>
<td>CD4</td>
<td>Positive on a minor subset of CD4 T-cells.</td>
<td>Positive. Slight dim expression in 50-80% of cases. Rare partial or complete negativity.</td>
</tr>
<tr>
<td>CD8</td>
<td>Positive on a minor subset of CD8 T-cells.</td>
<td>Positive. Slight dim expression in 10-50% of cases. Rare partial or complete negativity.</td>
</tr>
<tr>
<td>CD7</td>
<td>Variable expression on CD8 T-cells.</td>
<td>Variable expression in most cases.</td>
</tr>
<tr>
<td>CD28</td>
<td>Variable expression on CD4 T-cells.</td>
<td>Variable expression in most cases.</td>
</tr>
<tr>
<td>CD123a (KIR3DL2)</td>
<td>Largely negative on CD4 T-cells.</td>
<td>Positive in 10-20% of cases. Might depend on the antibody utilized.</td>
</tr>
<tr>
<td>CD144a (CD38)</td>
<td>Largely negative on CD4 T-cells.</td>
<td>Positive in 40-80% of cases. Slight dim expression inconsistently reported in 30% of cases.</td>
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<tr>
<td>CD144b (CD158k)</td>
<td>Positive on a subset of CD8 T-cells.</td>
<td>Variable or complete negativity in most cases.</td>
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<tr>
<td>CD279 (PD-L1)</td>
<td>Negative in 50% of cases.</td>
<td>Positive in 90-100% of cases.</td>
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Additional markers of potential diagnostic utility

- Naive-memory phenotypic markers:
  - CD45RA, CD27, CD117 (CSC/P), CD45RA, CD38, CD62L.
  - Sézary cells have a highly heterogeneous naive-memory phenotype.
  - The expression of these antigens in any particular case might be the most useful feature for gating.
- Therapeutic targets:
  - CD30, CD25, CD123, PD-L1.
  - No standard approach to assess the expression of these antigens and their clinical significance.
- Antigens relevant for other T-cell lymphoproliferative disorders:
  - CD2, CD3, CD4, CD10, CD19, CD25, CD30, CD56.
- Antigens relevant to other lymphoid subsets:
  - CD19, kappa, lambda.
- Antibody clone selection

- No specific reagent or vendor recommended.
  - Many commercially available reagents have been extensively tested and are likely to work well.
  - Supplementary information will be provided with list of reagents utilized by contributors to this consensus.
- The CD26 reagent should be carefully selected and titrated.
  - Some reported issues with signal-to-noise ratio.
Fluorochrome selection

- CD3, CD7, and CD26 should be matched with bright fluorochromes.
- CD4 and CD8 are typically brightly expressed and should fare well with moderately bright fluorochromes. However, prioritize CD4 over CD8.
- CD45 best coupled with dimmer and less discriminatory fluorochrome.

Assessment of Staining Adequacy

Goal

- CD3 and CD4 staining should discriminate slightly dim subsets from bright positive.
- CD7 and CD26 staining should discriminate dim or negative subsets from bright positive.

However...

- Current measures of staining adequacy do not adequately address the capacity to identify antigen loss.
- The dispersion of the log-normal fluorscence curve cannot be adequately described based on calculations provided by most analysis softwares.

Assessment of Staining Adequacy

Modified stain window

- Based on the mean and standard deviation of log-normal Gaussian curves.
- Estimates the width of the space between the curves, relative to the width of each curve, as visually appreciated on a log scale plot.
- Complicated formula due to log transformed data.

Preliminary plan for manuscript

- Provided and Excel sheet to calculate the SW based on conventional linear MFI and SD.
- Recommended method for CD3, CD4, and CD7 in at least 8 of 10 normal peripheral blood specimens.
- CD26 needs to be visually evaluated (no good normal positive control).

Specimen processing and event acquisition

Processing

- Wide variability in staining and lysing practices. No specific recommendations.
- Refer to ICCB quality standards. Module #1: Lysing Methods and Reagents for Flow cytometric immunophenotyping.

Acquisition

- Recommend minimum acquired events to approximate average of most reference institutions:
  - 20,000 lymphocytes
  - 100,000 leukocytes
  - 200,000 total events
- 500,000 might be required to detect low-level residual disease.
- Goal: Analytical sensitivity of 1% of white blood cells.

Gating strategies

Basic principles

- Contemporary approach to gating on lymphoid subsets.
- Identification of Sezary cells based on comprehensive immunophenotypic analysis (not just CD7 and CD26).
- Gating on Sezary cells based on the identification of an immunophenotypically abnormal cluster (different than normal).
- No specific template or gating order. Too much variability between analysis softwares and practice preferences.
Gating Strategy

The basics
- Exclude doublets and debris
- Gate on leukocytes and lymphocytes
- Time-based plot

Pitfalls:
- Tumor cells with high light scatter
- Tumor cells dim/negative for CD45
- Monocytes might be counted as abnormal lymphocytes or vice versa

Safeguards:
- Plot and/or back gate all CD3-positive events
- Confirm phenotype of monocytes

Gating Strategy

Gate on T and NK cells
- T-cells based on inclusive CD3 positivity
- NK cells based on CD3+CD7+(+)/CD3+/CD56+(+) or CD3+/CD7-CD56+(+)

Pitfalls:
- CD3 dim/negative T-cells might be missed or gated as NK cells.

Safeguards:
- Plot and/or back gate all CD4-positive lymphocytes
- Confirm phenotype of NK cells

Gating Strategy

Gate on CD4+ T-cells (and CD8+ T-cells)
- CD4+CD3 and CD8+CD3, or
- CD3-positive T-cells on CD4+CD8

Pitfalls:
- Monocytes may appear as CD4-dim
- Rare CD4+/CD8- or CD8+/Sezary cells

Safeguards:
- Confirm back gate CD4 dim events as monocytes
- Confirm phenotype CD8 T-cells
- Abnormal CD4+/CD8- or CD8+ subset may require additional work up

Gating Strategy

Gate on Sezary cells
- Plot CD4 T-cells on CD7vsCD26
- Examine clustered populations on other plots
- Examine abnormal subsets detected on other plots
- Plot CD7vsCD26

Pitfalls:
- CD7 and CD26 loss overlaps with reactive subsets
- Phenotypically complex Sezary cells might be underestimated

Safeguards:
- Always look for additional abnormalities besides loss of CD7 and/or CD26
- Consider prioritizing other abnormalities (dim CD3, dim CD4, high light scatter, dim CD45) over CD7/CD26 loss
Estimating absolute numbers of Sezary cells

Alternative methods

- **Dual platform based on mononuclear cells**
  - % abnormal cells of lymphocytes and monocytes x (ALC + AMC)
  - Overcomes loss of neutrophils and limitations of analyzer.
  - Strategy not yet tested.

- **Single platform (beads of volumetric)**
  - Direct quantitation of abnormal cells.
  - Lysis/wash might affect staining.
  - Not widely available / limited experience

- **Dual flow assay**
  - Second quantitative flow assay for lymphoid subsets (IVD or other).
  - Different processing and gating might result in different proportion of lymphoid subsets.

- Correlation with white blood cell counts of obtained separately from an automated blood analyzer.

- Correlation with absolute lymphocyte count (ALC)
  - % abnormal cells of lymphocytes x ALC
  - ALC might be falsely low due to large neoplastic cells counted as monocytes.
  - Risk of underestimating Sezary cells.

- Correlation with white blood cell count (WBC)
  - % abnormal cells of CD45+ leukocytes x WBC
  - Neutrophils and monocytes might be lost during some processing protocols.
  - Risk of overestimating Sezary cells.

- No agreement on a single method.
- Dual platform is currently the most commonly utilized method.
- Some propose that correlation with WBC should be considered first.
- Pitfalls of each method should be addressed during test validation.
The flow cytometry report

**Required elements**
- Presence of absence of abnormal T-cells
- Phenotype of abnormal T-cells
- Estimated absolute number of abnormal T-cells per µL of blood.
- Interpretation.

**Optional, depending on needs of clinical group**
- CD4:CD8 ratio
- Percentage of CD4 T-cells negative for CD7, CD26 and/or both.

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**Interpretation**:
An abnormal T-cell population detected. The abnormal T-cell population has abnormal expression of CD3(dim), CD7(dim to negative), CD26(absent), CD45(dim) with normal expression of CD2, CD4, CD5, and CD45; without CD8 or CD56. The abnormal population represents 12.2% of the total white cells. The population is consistent with previously diagnosed involvement by Sezary/Mycosis Fungoides.

Absolute clone size for CTCL like population: 0.68 thousand cells/microliter

Specimen Source: Peripheral Blood
Cell Concentration: 5.6 million/mL
CBC (12/4/2017) WBC: 5.4 K/uL
Specimen Viability: 98%
Specimen Quality Comment: Specimen adequate.

Flow Cytometry Antibodies Used: Analysis performed using anti-CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD45, CD56 and CD279 antibodies. Intensities of monoclonal antibodies not specifically mentioned above are within normal ranges. Number of antibodies used = 10.

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**Assessment of T-cell clonality**

Not a requirement, but might be useful in selected cases.

- TCR gene rearrangement study (molecular):
  - Biomed-2 primers.
  - Common false-positive results.
  - Subjective interpretation.
  - No direct correlation with phenotypic subset.

- TCR V-µ repertoire analysis (separate flow cytometry test):
  - IOTest Beta Mark TCR Vµ repertoire kit (Beckman Coulter).
  - 24 antibodies on 8 separate tubes, plus custom gating antibodies for each case.
  - High cost, demanding logistics and required expertise is a limiting factor.

- TCR C-β restriction (TRBC1 expression by flow cytometry):
  - Needs further study.

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**Flow cytometry report**

**Example of a positive result**

Interpretation:
No abnormal T-cell population detected.

T cells 5.2% of WBC

%CD4+CD3+ 62.9%
%CD8+CD3+ 29.5%
CD4/CD8 ratio: 2.1

CD4 positive CD7 negative 9.6 as % CD4
CD4 positive CD26 negative 39.7 as % CD4
CD4 positive CD7 and CD26 negative 6.4 as % CD4

Specimen Source: Peripheral Blood
Cell Concentration: 8.49 million/mL
CBC 01/25/2018 WBC: 8.4 K/uL
Specimen Viability: 97.5%
Specimen Quality Comment: Specimen adequate.

Flow Cytometry Antibodies Used: Analysis performed using anti-CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD45, CD56 and CD279 antibodies. Intensities of monoclonal antibodies not specifically mentioned above are within normal ranges. Number of antibodies used = 10.

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**Flow cytometry report**

**Example of a negative result**

Interpretation:
An abnormal T-cell population detected.

The abnormal T-cell population has abnormal expression of CD3(dim), CD7(dim to negative), CD26(absent), CD45(dim) with normal expression of CD2, CD4, CD5, and CD45; without CD8 or CD56. The abnormal population represents 12.2% of the total white cells. The population is consistent with previously diagnosed involvement by Sezary/Mycosis Fungoides.

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**Assay validation, optimization and ongoing quality monitors**

Work in progress

- ICCS quality standards will be extensively referenced.
- Validation of the qualitative and quasi-quantitative components of the test.
- Clinical and analytical sensitivity and specificity.
- Reproducibility.
- Reportable range.
Main Conclusions

• Identification and quantification of Sézary cells should be based on the identification of immunophenotypically abnormal T-cell subsets, based on comprehensive immunophenotypic analysis, and in alignment with contemporary flow cytometry practices.
  - Relying exclusively on CD7(+) and/or CD26(+) subsets on CD4+ T-cells is not recommended.

• At a minimum, a 6-color single tube analysis should be utilized, including:
  - CD3, CD4, CD7, CD8, CD26 and CD45.

• At a minimum, the report should include:
  - The presence or absence of an abnormal T-cell population.
  - A detailed phenotype of the abnormal population.
  - The calculated absolute number of abnormal cells/µL.
  - Interpretation.

• Several knowledge gaps are identified, needing further study.

Thank you!