Cytological preparations for molecular analysis-preanalytical issues for EBUS TBNA specimens: Cytospins

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Conflict of Interest

• **NO** CONFLICT OF INTEREST TO DISCLOSE
Introduction

• Cytospins are commonly produced cytological preparations

• Molecular potential not fully explored

  • Concentration of tumor cells
  • Multiples slides per sample
  • Ideal for FISH/ICC
    • Avoid nuclear truncation/whole cells

Targeted Use of Fluorescence In Situ Hybridization (FISH) in Cytospin Preparations

Results of 298 Fine Needle Aspirates of B-Cell Non-Hodgkin Lymphoma


- Use of cytospins from FISH in 6 years
- Successful results in 95.3% of the cases
  - Abnormalities found in 76%
- Use of CPs for accurately subtyping NHL
FISH in CPs


IgH/CCND1 fusion probe
MIB-1 in CPs

EBUS-TBNA specimens

- Subclassification of lymphomas using EBUS-TBNA samples
- 224 specimens
  - 10 lymphomas correctly subtyped
  - Use of cytomorphology, immunophenotyping, IHC and FISH

EBUS-TBNA specimens

Table I. Results of Ancillary Studies in Non-Hodgkin Lymphoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Cytologic diagnosis</th>
<th>Immunophenotyping</th>
<th>MIB1 PI (%)</th>
<th>FISH results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SLL/CLL</td>
<td>Kappa+, CD19+, dimCD20+, CD22−, FMC7−, CD5+, dimCD23+</td>
<td>2%</td>
<td>trisomy 12+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13q deletion+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11q deletion−</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17p deletion−</td>
</tr>
<tr>
<td>2</td>
<td>SLL/CLL with RS cells</td>
<td>Kappa+, CD19+, dimCD20+, FMC7−, CD5+, dimCD23+, CD10−</td>
<td>&lt;5%</td>
<td>trisomy 12+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13q deletion+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11q deletion−</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17p deletion−</td>
</tr>
<tr>
<td>3</td>
<td>MZL</td>
<td>Kappa+, CD19+, CD20+, CD22+, FMC7+, CD5−, CD10−, CD11c−</td>
<td>10%</td>
<td>ccnd1− trisomy 3−</td>
</tr>
<tr>
<td>4</td>
<td>LBCL</td>
<td>slg−, CD20+, CD79+, CD10+</td>
<td>60%</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>LBCL</td>
<td>Kappa+, CD20+, CD10+, CD19−, CD5−, FMC7−, CD23−, CD11c−</td>
<td>60%</td>
<td>(14;18)+ MYC-</td>
</tr>
<tr>
<td>6</td>
<td>LBCL (FL, G3)</td>
<td>Lambda+, CD19+, CD20+, CD79+, CD10+, CD23+, CD5−</td>
<td>40%</td>
<td>(14;18)+</td>
</tr>
<tr>
<td>7</td>
<td>Large cell lymphoma</td>
<td>failed</td>
<td>failed</td>
<td>N/A</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia; RS cells, Reed-Sternberg cells; MZL, marginal zone lymphoma; LBCL, large B cell lymphoma; FL, follicular lymphoma; G, grade; N/A, not applicable.

Table II. Results of Ancillary Studies and Histologic Confirmation in Hodgkin Lymphoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Cytologic diagnosis</th>
<th>Immunohistochemistry in RS cells</th>
<th>Clinical history</th>
<th>Histologic diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>HL</td>
<td>CD30+, CD15+, CD45−, CD20−</td>
<td>Primary</td>
<td>Classical HL, nodular sclerosis</td>
</tr>
<tr>
<td>9</td>
<td>HL</td>
<td>Insufficient tissue</td>
<td>Recurrence</td>
<td>Not done</td>
</tr>
<tr>
<td>10</td>
<td>HL</td>
<td>Insufficient tissue</td>
<td>Primary</td>
<td>Classical HL, nodular sclerosis</td>
</tr>
</tbody>
</table>

HL, Hodgkin lymphoma; RS cells, Reed-Sternberg cells.

EBUS-TBNA specimens

Fig. 2. A: CD30 positive R5 cell showing typical Golgi zone accentuation against a background of CLL cells (Cryolit block section immunohistochemistry, ×100). B: MIB1 staining in diffuse large B cell lymphoma (DLBCL) indicating a high proliferative rate (Cytopin immunohistochemistry, ×63). C: Cell surface marker immunophenotyping in DLBCL showing a mixture of normal T cells (~25%) and lambda light chain restricted, CD19+CD20+, CD10+, CD5– donal B cell population (linear scanning cytometry immunophenotyping). D: Interphase fluorescence in situ hybridization (FISH) showing trisomy 12 in B cell CLL (Cytopin CEP 12 FISH, ×100). (Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.)

Advantages of **cytospins** as source of DNA extraction

- Immediate visualization and morphologic assessment of samples
- High cellular samples when compared to cell blocks
- Higher quality DNA (no DNA degradation as seen in FFPE samples)

Chowdury et al. Optimizing the DNA yield for molecular analysis from cytologic preparations. *Cancer Cytopathology* 2016 Apr;124(4):254-60
<table>
<thead>
<tr>
<th>First author, year</th>
<th>Country where EGFR testing was performed</th>
<th>Patients, n (cytological samples)</th>
<th>Methods</th>
<th>Type of cytological samples</th>
<th>Type of preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellevicine, 2014</td>
<td>Italy</td>
<td>362</td>
<td>PCR + restriction fragment analysis</td>
<td>N/A</td>
<td>Smears, LBC</td>
</tr>
<tr>
<td>Hlinkova, 2013</td>
<td>Slovakia</td>
<td>679</td>
<td>HRMA</td>
<td>BB, PF, BW</td>
<td>N/A</td>
</tr>
<tr>
<td>Ma, 2012</td>
<td>Hong-Kong</td>
<td>269</td>
<td>PCR + Direct sequencing</td>
<td>FNA, PF, BB, BW, SP, others</td>
<td>CB</td>
</tr>
<tr>
<td>Mallapelle, 2013</td>
<td>Italy</td>
<td>305</td>
<td>PCR + restriction fragment analysis</td>
<td>FNA, fluids, BB, BW, SP</td>
<td>CB, smears</td>
</tr>
<tr>
<td>Peters, 2014</td>
<td>Australia</td>
<td>274*</td>
<td>PCR + Direct sequencing</td>
<td>FNA, PF, PCF</td>
<td>CB</td>
</tr>
<tr>
<td>Shiau, 2014</td>
<td>Canada</td>
<td>513</td>
<td>PCR + restriction fragment analysis</td>
<td>BW, BB, FNA, PF</td>
<td>CB</td>
</tr>
<tr>
<td>Wu, 2014</td>
<td>China</td>
<td>434</td>
<td>Real time PCR</td>
<td>FNA, BB, BAL, PF, SP, EBUS-TBNA</td>
<td>LBC</td>
</tr>
</tbody>
</table>
Extraction methods from archived cytospins and smears

- Classically described
- Good results using archived specimens


High-throughput panel

- High throughput multiplex mutation analysis using multiple cytological preparations
- Pre-designed MASS-ARRAY spectrometry for specific DLBCL mutations

Residual Material from Non-Hodgkin B-cell Lymphomas

+ Additional Samples (previous, later and concurrent) from the cases

FTA® Card

Manufacturer’s protocol for DNA extraction

FFPE tissue

Manual protocol

Archived smears

Archived cytospins

Frozen tissue

Qiagen© DNAeasy protocol

Sequenom© MASS-ARRAY Spectrometry

Positive and Failed cases

Direct Sequencing
Total DNA yield and successful rate of different types of sample

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>n $</th>
<th>DNA concentration (range)</th>
<th>% of successful results using MASS-ARRAY spectometry *</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTA® Card</td>
<td>80</td>
<td>6.44 (0.89-21.89)</td>
<td>99.7</td>
</tr>
<tr>
<td>Frozen tissue</td>
<td>13</td>
<td>23.32 (2.7-115.6)</td>
<td>100</td>
</tr>
<tr>
<td>FFPE</td>
<td>3</td>
<td>10.1 (5.6-14.7)</td>
<td>91.6</td>
</tr>
<tr>
<td>Smears</td>
<td>23</td>
<td>6.69 (0.38-26.87)</td>
<td>65.2</td>
</tr>
<tr>
<td>Cytospins</td>
<td>4</td>
<td>4.75 (3.2-6.3)</td>
<td>75</td>
</tr>
</tbody>
</table>
Conclusions

• Mutational status of samples varied in one third of the positive cases ➔ Patients should be treated according to their current molecular findings, and not based on results obtained from previous specimens

• Successful and reliable results were obtained using residual material from FNAs stored in FTA® cards as well as from smears and cytopsins, using high-throughput multiplex mutation analysis followed by direct sequencing.

• Effective use of minimal, limited specimens increase the potential number of samples available for molecular analysis.

Conclusions

• One of the limitations of the FTA card is the lack of cellular assessment.

• **Cytospins** can be produced with 150µl of needle rinse and used for cell count.

Use of cytospins

- High throughput multiplex mutation analysis using archived cytospins
- Cases of B-cell NHL previously tested for MYC and IGH/BCL2 translocations

Remaining archived cytospins (kept at -20°C)

100 µl of a 4:1 solution of buffer ATL and proteinase K

Tissue lysis overnight at 60°C

Qiagen® DNAeasy protocol

Sequenom© MASS-ARRAY Spectometry

5 assays at the same time:
- second position of codon 265 of MYD88
- first and second positions of codon 196 of CD79B
- first and second positions of codon 641 of EZH2


Positive and Failed cases: Direct sequencing
Results

Median number of slides used per case: 2.5 (1-4)

Average DNA concentration: 9.46ng/µl

Archived at -20°C from 2 to 6 years

Range 1.88 to 62.85ng/µl

88% successful results

100% successful results
Conclusions

• CPs were shown to be a reliable source of high-quality genomic material with successful results in cases stored for up to six years.
• Almost all B-cell NHL with point mutations showed concurrent chromosomal abnormalities.
• Effective use of minimal, limited specimens increase the potential number of samples available for molecular analysis.
Cell enrichment of cytospins

- Uses microdissection with MultiTech DNA extraction solution
  - Better visualization of cells, better quality of DNA
- EGFR analysis using multiple techniques
- Successful results using preparations (including cytospins) with as little as 25 tumor cells

Oh SY, Lee HT.
Efficiency of EGFR mutation analysis for small microdissected cytological specimens using multitech DNA extraction solution. Cancer Cytopathology 2015 Jul;123(7):401-12
More pre-analytical issues

- Uses cytospins from lung cancer cell lines and from clinical cases
- Compares two types of methods for cell extraction, from different types of glass slides

Chowdury et al. Optimizing the DNA yield for molecular analysis from cytologic preparations. Cancer Cytopathology 2016 Apr;124(4):254-60
Comparison of methods of cell extraction and type of glass slides

Figure 2. Comparing the DNA yields of cell-line cytospin preparations from FF and NF slides and from FF and PC slides with either scalpel-blade scraping or cell lifting as the tissue-extraction methodology. Error bars indicate ±1 standard deviation. FF indicates fully frosted; NF, nonfrosted; PC, positively charged.

Chowdury et al. Optimizing the DNA yield for molecular analysis from cytologic preparations. Cancer Cytopathology 2016 Apr;124(4):254-60
Issues raised with the use of cytospins

• Low cellular samples → Increase the number of slides

• Use of archived material → Whole-slide imaging/scanning

• Type of slide → prefer positively charged over frosted
# Types of preparations

<table>
<thead>
<tr>
<th>Cytologic Substrate</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct smear</td>
<td>• Immediate assessment for adequacy</td>
<td>• Sacrificing slide from archival material (potential medicolegal issues)</td>
</tr>
<tr>
<td></td>
<td>• High-quality nucleic acid</td>
<td>• Additional validation</td>
</tr>
<tr>
<td></td>
<td>• Whole cells with whole nuclei</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Superior tumor mapping in samples with low tumor fraction</td>
<td></td>
</tr>
<tr>
<td>Liquid-based cytology</td>
<td>• Standardized processing with optimal preservation of cells</td>
<td>• Lack of immediate assessment</td>
</tr>
<tr>
<td></td>
<td>• Whole cells with whole nuclei</td>
<td>• Additional validation</td>
</tr>
<tr>
<td></td>
<td>• High-quality nucleic acid</td>
<td>• Nucleic acid retrieval may be variable based on preservative/fixative</td>
</tr>
<tr>
<td>Cell block</td>
<td>• Ease of acquisition</td>
<td>• Lack of immediate assessment</td>
</tr>
<tr>
<td></td>
<td>• Multiple serial sections</td>
<td>• Frequently suboptimal cellularity</td>
</tr>
<tr>
<td></td>
<td>• Standardized validation in most molecular laboratories</td>
<td>• Nucleic acid may be suboptimal because of formalin fixation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Partial nuclei on standard 4- to 5-micron sections</td>
</tr>
</tbody>
</table>

TAKE HOME MESSAGES

• Addition of all sorts of cytological preparations increase the options of samples for molecular testing (avoid repeat biopsy)

• Results and pre-analytical variables similar to smears

• Needs optimization and standardization, especially when dealing with modern technologies (NGS)
Thank you!