Needles and Haystacks
Implications of ALK translocations as biomarkers, and novel approaches in a setting of routine, high throughput lung cancer screening

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Principal Scientist - Oncology
Quest Diagnostics Nichols Institute
Background and Objectives

- Overview of current pathways in NSCLC
- Review diagnostic testing options
- Implications of *EML4-ALK* translocations as biomarkers for screening in drug development and clinical trial programs
- Explore the optimal use of *EML4-ALK* assays for patient screening
## NSCLC Overview - Standard Treatments

### Standard Treatment Options

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment Options</th>
<th>Key Issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - II</td>
<td>Curative Lung Resection</td>
<td>Non-specific targeting of tumor cells</td>
</tr>
<tr>
<td>III (locally advanced)</td>
<td>Combined Chemoradiotherapy</td>
<td>Modest improvement in mortality over standard supportive care (average 2 months’ prolonged survival)</td>
</tr>
<tr>
<td>IIBB – IV (advanced metastatic)</td>
<td>Systemic platinum-based therapy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Combined platinum (cisplatin or carboplatin)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>New generation chemotherapeutics (paclitaxel, docetaxel, gemcitabine)</td>
<td></td>
</tr>
</tbody>
</table>
## NSCLC Overview - Targeted Treatments

### Targeted Treatment Options

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Treatment Options</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (angiogenesis)</td>
<td>Bevacizumab (Avastin™)</td>
<td>Targets oncogenic molecular pathways</td>
</tr>
<tr>
<td>EGFR</td>
<td>Gefitinib (Iressa™)</td>
<td>May help enhance clinical efficacy while minimizing toxicity</td>
</tr>
<tr>
<td></td>
<td>Erlotinib (Tarceva™)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cetuximab (Erbitux™)</td>
<td>Requires accurate screening for optimal effect</td>
</tr>
</tbody>
</table>
Mutation Frequencies by NSCLC Subtype

**Adenocarcinoma (35-40%)**
- EGFR: 38%
- KRAS: 22%
- Other: 25%
- PIK3CA: 3%
- BRAF: 4%
- EML4-ALK: 7%
- PTEN: 1%

**Squamous cell carcinoma 25-30%**
- EGFR: 4%
- KRAS: 6%
- EML4-ALK: 1%
- BRAF: 2%
- PIK3CA: 3%
- Other: 76%
- PTEN: 8%

**Large Cell (10-15%)**
- EGFR: 5%
- KRAS: 15%
- PIK3CA: 6%
- Other: 74%
Overview of EGFR-Related Pathways

Intracellular Signaling

Receptor Tyrosine Kinases

- ALK
- EGFR
- MET

Frizzled

- PI3K
- AKT
- mTOR
- MDM2

- KRAS
- BRAF
- MEK
- MAPK
- β-catenin

Nuclear Signaling

- p16
- Rb
- p53
- p16
- Rb
Patients | Growth | People

Oncogenes Known to Have Mutations in NSCLC

Tumor Suppressors

- PTEN
- LBKB1
- p53
- p16
- Rb

Receptor Tyrosine Kinases

- ALK
- EGFR
- MET
- Frizzled

EML4-ALK activates multiple pathways independent of upstream EGFR TK activity

Intracellular Signaling

- PI3K
- AKT
- mTOR
- MDM2
- KRAS
- BRAF
- MEK
- MAPK

Nuclear Signaling

- β-catenin

Intracellular Signaling and Nuclear Signaling pathways are interconnected.
Implications for Targeted Therapeutics

Tumor Suppressors

- **ALK** → PI3K → AKT → mTOR → MDM2
- **EGFR** → KRAS → BRAF → MEK → MAPK
- **MET** → Frizzled → β-catenin

**Receptor Tyrosine Kinases**

- **PTEN** → LBKB1 → p53 → p16 → Rb

**Intracellular Signaling**

**Nuclear Signaling**

- R Resistance shown in NSCLC

Patients | Growth | People
Implications for Targeted Therapeutics

- **ALK**, **EGFR**, **MET** shown effective in NSCLC in clinical trials


- Receptor Tyrosine Kinases
- Intracellular Signaling
- Nuclear Signaling

- Resistance shown in NSCLC
- Shown effective in NSCLC in clinical practice

- Shown effective in NSCLC in clinical trials

- Patients | Growth | People
Mutation Incidence in NSCLC
Adenocarcinoma

Adenocarcinoma (35-40%)

- Smokers
  - EGFR 38%
  - KRAS 22%
  - EML4-ALK 7%
  - PIK3CA 3%
  - PTEN 1%
  - Other 25%

- Non-smokers
  - EML4-ALK 7%
  - PIK3CA 3%
  - BRAF 4%
  - Other 25%

Mutation testing scheme...

Lung Cancer Mutation Panel

- EGFR
- KRAS
- EML4-ALK
- BRAF
- PIK3CA
- NRAS/HRAS
Mutation Incidence in NSCLC Adenocarcinoma

Adenocarcinoma (35-40%)

- EGFR: 38%
- KRAS: 22%
- EML4-ALK: 7%
- Other: 25%
- PTEN: 1%
- PIK3CA: 3%
- BRAF: 4%

Primary or acquired resistance?

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rx Resistance</th>
<th>Gefitinib</th>
<th>Erlotinib</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR</strong></td>
<td>T790M substitution</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>E796A substitution</td>
<td>R</td>
<td>Unk</td>
</tr>
<tr>
<td></td>
<td>E884K substitution</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><strong>c-Met</strong></td>
<td>Amplification</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Mutation testing scheme...

Lung Cancer Mutation Panel
### Treatment Selection Testing Capabilities through Quest Diagnostics

<table>
<thead>
<tr>
<th>Treatment Indications</th>
<th>Quest Diagnostics Tests</th>
<th>Response Indication</th>
<th>NSCLC Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR TKIs/anti-EGFR</td>
<td><em>EGFR</em> mutations by sequencing (exons 18-21)</td>
<td>Sensitivity/Resistance</td>
<td>5% - 40%*</td>
</tr>
<tr>
<td></td>
<td><em>EGFR</em> mutations by DxS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>EGFR</em> amplification by FISH</td>
<td>Sensitivity</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>c-Met</em> amplification by FISH/IHC</td>
<td>Resistance</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> mutations by sequencing (exons 1-2)</td>
<td>Resistance</td>
<td>6% - 25%*</td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> mutations by DxS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>ALK1</em> rearrangement by FISH/IHC/IDE (qRT-PCR)</td>
<td>Resistance</td>
<td>3-7%</td>
</tr>
<tr>
<td></td>
<td><em>EML4-ALK</em> by RT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>BRAF</em> mutations by sequencing (exons 11, 12, 15)</td>
<td>Resistance</td>
<td>2-4%</td>
</tr>
<tr>
<td></td>
<td><em>PIK3CA</em> mutations by sequencing (exons 1, 9, 20)</td>
<td>Resistance</td>
<td>3-6%</td>
</tr>
<tr>
<td></td>
<td><em>NRAS</em> mutations by sequencing (exons 1-2)</td>
<td>Resistance</td>
<td>Rare</td>
</tr>
<tr>
<td></td>
<td><em>HRAS</em> mutations by sequencing (exons 1-2)</td>
<td>Resistance</td>
<td>Rare</td>
</tr>
<tr>
<td></td>
<td><em>HER2</em> amplification by FISH</td>
<td>Resistance</td>
<td>Rare</td>
</tr>
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<td>ALK inhibitors</td>
<td><em>ALK1</em> rearrangement by FISH/IHC/IDE (qRT-PCR)</td>
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<tr>
<td></td>
<td><em>EGFR</em> mutations by DxS (29 mutations)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> mutations by sequencing (exons 1-2)</td>
<td>Resistance</td>
<td>6% - 25%*</td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> mutations by DxS (7 mutations)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Incidence largely dependent on histologic subtype and/or smoking history

*Listed Tests Currently Validated or In Validation Process*
What Is *EML4-ALK* and How Do We Test for It?

**Description**

- EML4-ALK is a fusion product that results from various rearrangements of the *EML4* and *ALK* genes.
- Similar rearrangements are present in subsets of lymphomas. Many laboratories test for these using FISH.
- There are >15 different fusion transcript variants, and most can be detected by FISH. However, interpretation is more difficult than for other FISH assays.
Implications for Pharma

- ~3-7% of NSCLC patients are positive for *EML4-ALK*.
- Using FISH for detection in large-scale population screening becomes laborious and expensive.
- Patient selection for clinical trials to date has been done with FISH testing, but there is no clinical standard yet. Neither method can be considered superior for patient selection.

Screening out negative patients could eliminate unnecessary, expensive testing in >90% of cases.
## Treatment Selection Testing Capabilities through Quest Diagnostics

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<td></td>
</tr>
<tr>
<td></td>
<td>EML4-ALK by RT-PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALK IDE (qRT-PCR)</td>
<td></td>
</tr>
</tbody>
</table>

**Response Indication**

- ALK1 by FISH: Resistance
- ALK1 by IHC: Sensitivity

**Listed Tests Currently Validated or In Validation Process**

- FISH
- IHC
- RT-PCR w/ fragment analysis
- IDE by qRT-PCR

Patients | Growth | People
Large Scale Screening Solutions
Intragenic Differential Expression of ALK
Chromosome 2p Inversion Leads to **EML4-ALK** Fusion

- **EML4** gene transcription
- **ALK** gene transcription
- **Microtubule Formation**
- **Cell Growth & Proliferation**

**EML4-ALK** gene transcription
Chromosome 2p Inversion Leads to \textit{EML4-ALK} Fusion

**EML4:ALK Fusion Negative Cells**

- \(5'\)-ALK
- ALK
- \(3'\)-ALK

**EML4**

**Lung Cancer Cell with EML4:ALK Fusion**

- \(5'\)-ALK
- EML4
- ALK
- \(3'\)-ALK

**EML4:ALK Fusion Positive**

\(\text{IDE} = \Delta \text{Exp}\)

5'ALK 3'ALK

*Patent pending*
Identifying ALK Translocations via RT-PCR

**Specimen**
- **Specimen type**
  - Tumor
  - Biopsy
  - Pleural effusion
  - Plasma

**RNA Extraction**
- **Quantitative RT-PCR**
  - Reference Gene
  - Multiplex ALK

- **Negative Intragenic Differential Expression**

- **Positive Intragenic Differential Expression**

- 3 – 7% Incidence

- **40 Samples per plate**
Confirmatory and Routine Testing

Detection of *EML4-ALK* by Fluorescence in situ Hybridization (FISH), Immunohistochemistry (IHC) and Direct RT-PCR
Detection of 2p23 Rearrangements Involving ALK1 by FISH in Lung Cancer FFPE Tissue

**Figure.** Fluorescent images of cells from formalin-fixed paraffin-embedded lung tissue hybridized with LSI® ALK Dual Color rearrangement probe (Abbott/Vysis, Abbott order no. 05J89-001). The normal (A) signal pattern is 2 fusion (2F), negative for rearrangement of ALK (green arrows). The abnormal (B) signal pattern is 1R1G1F, positive for rearrangement of ALK (magenta arrows).
Detection of ALK expression by immunohistochemistry (IHC)

- CONFIRM™ anti-ALK1 Primary Antibody: Targets NPM-ALK fusion protein found in anaplastic large cell lymphoma (ALCL)

- Recognition mainly in ALK TK domain; it also detects other ALK fusion proteins, including EML4-ALK, in NSCLC

- IHC staining in NSCLC FFPE tissue to identify positive EML4-ALK positive with anti-ALK1 (left) vs non-immune (right)
Cross Validation Using Molecular Detection FISH and IHC

- One sample tested negative in FISH but positive by IDE (qRT-PCR)
- Additional IHC analysis to evaluate FISH negative samples

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK IDE</td>
<td>5</td>
<td>62</td>
</tr>
<tr>
<td>FISH</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td><strong>Concordance</strong></td>
<td><strong>&gt; 98%</strong></td>
<td></td>
</tr>
</tbody>
</table>

- IHC staining in NSCLC FFPE tissue to identify positive EML4-ALK 3a/b variant positive with anti-ALK1 (left) vs non-immune (right)

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK IDE</td>
<td>6</td>
<td>65</td>
</tr>
<tr>
<td>IHC</td>
<td>5</td>
<td>66</td>
</tr>
<tr>
<td><strong>Concordance</strong></td>
<td><strong>&gt; 98%</strong></td>
<td></td>
</tr>
</tbody>
</table>
Direct RT-PCR Detection of EML4-ALK in Lung Cancer FFPE Tissue

EML4-ALK fusion transcript variants identified to date

- EML4 exons
- ALK exons
- EML4 or ALK partial introns

Transcription start site → Forward primers → Reverse FAM-labeled primer

### Cross Validation Examples for EML4-ALK Test Menu

<table>
<thead>
<tr>
<th>Sample</th>
<th>ALK IDE (qRT-PCR)</th>
<th>EML4-ALK Fusion (RT-PCR)</th>
<th>ALK1 Immunohistochemistry</th>
<th>ALK1 FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>107114BB EML4-ALK Negative</td>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
<td><img src="image3.jpg" alt="Image" /></td>
<td><img src="image4.jpg" alt="Image" /></td>
</tr>
<tr>
<td>ONK3993 EML4-ALK Variant 8a/b</td>
<td><img src="image5.jpg" alt="Image" /></td>
<td><img src="image6.jpg" alt="Image" /></td>
<td><img src="image7.jpg" alt="Image" /></td>
<td><img src="image8.jpg" alt="Image" /></td>
</tr>
<tr>
<td>11413958 EML4-ALK Variant 1</td>
<td><img src="image9.jpg" alt="Image" /></td>
<td><img src="image10.jpg" alt="Image" /></td>
<td><img src="image11.jpg" alt="Image" /></td>
<td><img src="image12.jpg" alt="Image" /></td>
</tr>
<tr>
<td>PA0F3367C4 EML4-ALK Variant 3a/b</td>
<td><img src="image13.jpg" alt="Image" /></td>
<td><img src="image14.jpg" alt="Image" /></td>
<td><img src="image15.jpg" alt="Image" /></td>
<td><img src="image16.jpg" alt="Image" /></td>
</tr>
<tr>
<td>PA15E7F7AF3 EML4-ALK Variant 3a/b</td>
<td><img src="image17.jpg" alt="Image" /></td>
<td><img src="image18.jpg" alt="Image" /></td>
<td><img src="image19.jpg" alt="Image" /></td>
<td><img src="image20.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>
## Relative Benefit by Test

<table>
<thead>
<tr>
<th>Test</th>
<th>Routine/ Familiarity</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Time/Assay</th>
<th>Throughput</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK by FISH</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ALK by IHC</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>EML4-ALK by Direct RT-PCR</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>ALK by IDE (qRT-PCR)</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>
• Molecular testing for gene mutations continues to become more relevant as a biomarker in a clinical setting.

• Quest Diagnostics has a comprehensive mutation detection menu to keep pace with the current needs in diagnostics.

• *EML4-ALK* has proven to be a highly relevant new biomarker for NSCLC.

• Multiple methods are available for *EML4-ALK* detection.
• For specific \emph{EML4-ALK} variant detection, direct RT-PCR is the only option.

• Large population-based screening can be made more efficient by pre-screening to eliminate further testing in \emph{EML4-ALK}–negative cases.

• ALK IDE is a robust, high-throughput tool for pre-screening and may prove to be sufficient for conclusive determination of \emph{EML4-ALK} status.
## Calculation and interpretation

<table>
<thead>
<tr>
<th>Ln(3’ALK/5’ALK)</th>
<th>Ln(3’ALK/ABL)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2.1</td>
<td>Any</td>
<td>Rearrangement Not Detected</td>
</tr>
<tr>
<td>&gt; 2.1 but &lt; 3.0</td>
<td>AND &lt; 0.06</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>&gt; 2.1</td>
<td>AND &gt; 0.06</td>
<td>Rearrangement Detected</td>
</tr>
<tr>
<td>&gt; 3.0</td>
<td>Any</td>
<td>Rearrangement Detected</td>
</tr>
<tr>
<td>Method</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>ALK IDE</td>
<td>6</td>
<td>102</td>
</tr>
<tr>
<td>Direct RT-PCR</td>
<td>6</td>
<td>102</td>
</tr>
<tr>
<td><strong>Concordance</strong></td>
<td><strong>100%</strong></td>
<td></td>
</tr>
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</table>
One sample tested negative by IHC but positive by IDE (qRT-PCR)
Additional RT-PCR analysis to evaluate the FISH and IHC negative Samples

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<tr>
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</thead>
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<td><strong>Concordance</strong></td>
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One sample tested negative by IHC but positive by IDE (qRT-PCR) and direct RT-PCR

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