DIAGNOSTIC WORK UP IN NSCLC AND THE IMPORTANCE OF OPTIMAL TISSUE MANAGEMENT IN THE ERA OF PRECISION MEDICINE

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Hospital Clinic Barcelona, Barcelona, Spain
OUTLINE

- Precision medicine in advanced NSCLC
- Updated ESMO Guidelines for NSCLC genomic testing (2020)
- Optimal Diagnostic Workup of lung cancer:
  - Sample Journey
  - Morphology & Histology
  - Type of samples for Genomic Evaluation
  - Input requirements & Quality Control for Biomarker Testing
- Guidelines recommendations: PD-L1, \textit{EGFR, ALK, ROS1, BRAF, NTRK}
- Other actionable predictive biomarkers: \textit{RET, MET, KRAS, HER2, EGFR\textsubscript{ins20}}
- NGS
- Summary / Conclusions
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NSCLC MOLECULAR CLASSIFICATION

NSCLC classification has moved from histologic to molecular subtyping

1990

NSCLC as one disease

1990

Adenocarcinoma 60%

Squamous 20%

Others 20%

2004

Adenocarcinoma 60%

2022

Unknown

KRAS

EGFR

ALK

BRAF

MET

HER2

PI3K

ROS1

NTRK

RET

MAPK

NRG

Unknown

Molecular Testing: Adenocarcinoma and NSCLC-NOS histologies

NSCLC, non-small cell lung cancer; NOS, not otherwise specified

Li T et al. J Clin Oncol 2013; 31:1039-1049
## DRIVER ONCOGENES IN LUNG ADENOCARCINOMA BY ETHNICITY

<table>
<thead>
<tr>
<th>Genes</th>
<th>USA/Europe</th>
<th>East Asia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR</strong></td>
<td>5-19.4%</td>
<td>40-59%</td>
</tr>
<tr>
<td><strong>KRAS</strong></td>
<td>20-30%</td>
<td>7.4-11%</td>
</tr>
<tr>
<td><strong>ALK</strong></td>
<td>3-6%</td>
<td>3-7%</td>
</tr>
<tr>
<td><strong>ROS1</strong></td>
<td>1-2%</td>
<td>1-3%</td>
</tr>
<tr>
<td><strong>BRAF</strong></td>
<td>2-3%</td>
<td>0.5-1%</td>
</tr>
<tr>
<td><strong>RET</strong></td>
<td>1-2%</td>
<td>1-2%</td>
</tr>
<tr>
<td><strong>MET</strong></td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td>2-3%</td>
<td>2-3%</td>
</tr>
<tr>
<td><strong>NTRK</strong></td>
<td>0.23%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

More than 50% of all lung adenocarcinomas harbour driver oncogenes. Incidence of genomic driver is variable among ethnic populations.
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METASTATIC NSCLC: ESMO CLINICAL PRACTICE GUIDELINES

Stage IV Non-Squamous Cell Carcinoma

A. Molecular Tests Positive (ALK/BRAF/EGFR/ROS1)

B. Molecular Tests Negative (PD-L1 expression)

Stage IV Squamous Cell Carcinoma

LoE, Level of evidence (I-V); GoR, grade of recommendation (A-E).

Planchar D, et al. Ann Oncol 2018;29(Supplement_4):iv192–iv237, © 2018 European Society for Medical Oncology. Published by Elsevier Ltd. All rights reserved.

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**Molecular Pathology/Biology ESMO Testing Guideline Recommendations**

**2019 NSCLC Recommendations**

1. **‘Must-test’ Predictive Biomarkers**
   - EGFR
   - ALK
   - ROS1
   - BRAF
   - PD-L1

2. **‘Should-test’ Emerging Biomarkers**
   - KRAS
   - RET
   - NTRK
   - MET
   - HER2

**2021 NSCLC Recommendations**

1. **‘Must-test’ Predictive Biomarkers**
   - EGFR
   - ALK
   - ROS1
   - BRAF
   - NTRK
   - PD-L1

2. **‘Should-test’ Emerging Biomarkers**
   - KRAS
   - RET
   - HER2
   - MET

Exp, expression; mut, mutation; NSCLC, non-small cell lung cancer; rearr, rearrangement

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**Biomarker testing**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Method</th>
<th>Use</th>
<th>LoE, GoR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR mutation</strong></td>
<td>Any appropriate, validated method, subject to external quality assurance</td>
<td>To select those patients with <em>EGFR</em>-sensitising mutations most likely to respond to <em>EGFR</em> TKI therapy</td>
<td>I, A</td>
</tr>
<tr>
<td><strong>ALK rearrangement</strong></td>
<td>Any appropriate, validated method, subject to external quality assurance. FISH is the historical standard but IHC is now becoming the primary therapy-determining test, provided the method is validated against FISH or some other orthogonal test approach. NGS is an emerging technology</td>
<td>To select those patients with <em>ALK</em> gene rearrangements most likely to respond to <em>ALK</em> TKI therapy</td>
<td>I, A</td>
</tr>
<tr>
<td><strong>ROS1 rearrangement</strong></td>
<td>FISH is the trial-validated standard. IHC may be used to select patients for confirmatory FISH testing but currently lacks specificity. NGS is an emerging technology. External quality assurance is essential</td>
<td>To select those patients with <em>ROS1</em> gene rearrangements most likely to respond to <em>ROS1</em> TKI therapy</td>
<td>II, A</td>
</tr>
<tr>
<td><strong>BRAF mutation</strong></td>
<td>Any appropriate, validated method, subject to external quality assurance</td>
<td>To select those patients with <em>BRAF</em> V600-sensitising mutations most likely to respond to <em>BRAF</em> inhibitor, with or without MEK inhibitor therapy</td>
<td>II, A</td>
</tr>
<tr>
<td><strong>NTRK rearrangement</strong></td>
<td>Screening by IHC or RNA NGS. A positive with the former requires confirmation by a molecular method (FISH, NGS). The latter should probably be validated by IHC</td>
<td>To select those patients with <em>NTRK</em> gene rearrangements most likely to respond to <em>NTRK</em> TKI therapy</td>
<td>II, A</td>
</tr>
<tr>
<td><strong>PD-L1 expression</strong></td>
<td>IHC to identify PD-L1 expression at the appropriate level and on the appropriate cell population(s) as determined by the intended drug and line of therapy. Only specific trial assays are validated. Internal and external quality assurance are essential.</td>
<td>To enrich for those patients more likely to benefit from anti-PD-1 or anti-PD-L1 therapy. For pembrolizumab, testing is a companion diagnostic for nivolumab and atezolizumab, testing is complementary.</td>
<td>I, A</td>
</tr>
</tbody>
</table>

LoE, Level of evidence (I-V); GoR, grade of recommendation (A-E); TKI, tyrosine-kinase inhibitor; IHC, immunohistochemistry; FISH, fluorescent in situ hybridization; NGS, next generation sequencing.
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Before starting, review relevant clinical information such as:
- Sample site
- Clinical suspicion on primary lung tumour or metastasis
- Reason for biopsy: diagnosis, molecular evaluation, resistance
- Smoking history

Then, establish diagnostic work up:
- **FIRST**  ➔ Establish malignancy
- **SECOND**  ➔ Define type of malignancy
- **THIRD**  ➔ Perform molecular testing as appropriate and prioritise testing according to individual patients
Type of malignancy

<table>
<thead>
<tr>
<th>TUMOUR TYPE</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma (ADC)</td>
<td>CK7, TTF-1</td>
</tr>
<tr>
<td>Squamous cell carcinoma (SCC)</td>
<td>P40 (P63, less specific)</td>
</tr>
<tr>
<td>Neuroendocrine (LCNEC, SCLC)</td>
<td>CD56 (NSE, chromogranin, synaptophysin)</td>
</tr>
</tbody>
</table>

1. IHC should not be performed unless necessary
2. In NSCLC-NOS, IHC reduces the number of inconclusive diagnoses (rate < 10%)
3. Limiting IHC between 2 and 4 markers seems reasonable

In NSCLC biopsy samples the following terminology should be used:

- ADC IHC not required if diagnostic morphology present
- SCC IHC not required if diagnostic morphology present
- Carcinomas lacking clear differentiation by morphology and special stains are classified as NSCLC-NOS in small biopsies/cytology specimens and as Large Cell Carcinomas in resection specimens
MORPHOLOGY & HISTOLOGY: IMMUNOHISTOCHEMICAL MARKERS

Type of malignancy

Lung adenocarcinoma, TTF1 positive

Squamous Cell Carcinoma, P40 positive

Courtesy of Dr. N. Vidal, Pathology Department, Hospital Clinic Barcelona
CITOLOGY FOR MOLECULAR DIAGNOSTICS

- 40% NSCLC diagnosed by cytology

GENERAL COLLECTION TECHNIQUES

Smear  ThinPrep  CytoSpin  Cell block

May use a variety of stains: PAP, diff quick, H&E, etc.

The Reality Sample Size  Ideal Sample Size

H&E: hematoxylin and eosin stain; NSCLC, non-small cell lung cancer; PAP, papanicolaou

From files of Teixidó C, Reguart N
INPUT REQUIREMENTS FOR BIOMARKER TESTING

Tissue requirements

- H&E
- IHC
- FISH
- RT-PCR
- NGS

H&E: hematoxylin and eosin stain; IHC: immunohistochemistry; FISH, fluorescent in situ hybridization; RT-PCR, reverse transcription polymerase chain reaction; NGS, next generation sequencing

* Numbers are approximate and tissue requirements depend on technique/reagent/platform

From files of Teixidó C, Reguart N
QUALITY CONTROL FOR BIOMARKER TESTING

- The molecular specialist must evaluate whether the percentage of tumour in a given specimen reaches the detection threshold of the specific molecular test or tests.

Assess tumour percentage and viability

Formalin-fixed paraffin embedded (FFPE) samples

Cytology samples

DQ: diff quick; H&E: hematoxylin and eosin stain; T, tumour; ADC, adenocarcinoma
STRATEGY TO MAXIMISE TISSUE FOR MOLECULAR TESTING

H&E for Histological Diagnosis

Repeat sectioning

For IHC

H&E

For Molecular Testing

At the time of initial sectioning for morphologic diagnosis, additional sections may be taken, in anticipation of IHC or predictive markers analyses

Molecular testing sections can be prepared:
Together with first H&E section
After initial H&E for histological diagnosis

The Reality Sample Size

Ideal Sample Size

H&E: hematoxylin and eosin stain; IHC, immunohistochemistry

From files of Teixidó C, Reguart N
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PD-L1
Programmed Cell Death Ligand-1
PD-L1 TESTING FOR IMMUNOTHERAPY

Standard Detection by IHC

- PD-L1 expression has been detected on tumour cells and tumour-infiltrating immune cells
- PD-L1 on tumour cells may lead to the inhibition of activated T-cells
- To enrich for those patients more likely to benefit from anti-PD-1 or anti-PD-L1 therapy
- IHC to identify PD-L1 expression at the appropriate level and on the appropriate cell population(s) as determined by the intended drug and line of therapy

From files of Teixidó C, Reguart N

IHC: immunohistochemistry
# PD-L1 IHC ASSAYS IN LUNG CANCER

Summary of PD-L1 antibodies and technical aspects for evaluation in NSCLC

<table>
<thead>
<tr>
<th>mAb clone</th>
<th>Ab host species</th>
<th>Platform</th>
<th>PD-L1 scoring</th>
<th>Cut-offs</th>
</tr>
</thead>
<tbody>
<tr>
<td>22C3</td>
<td>Mouse</td>
<td>Dako</td>
<td>TC</td>
<td>TC ≥1% or TC ≥50%</td>
</tr>
<tr>
<td>28-8</td>
<td>Rabbit</td>
<td>Dako</td>
<td>TC</td>
<td>TC ≥1%</td>
</tr>
<tr>
<td>SP142</td>
<td>Rabbit</td>
<td>Ventana</td>
<td>TC, IC</td>
<td>TC ≥50% or IC ≥10%</td>
</tr>
<tr>
<td>SP263</td>
<td>Rabbit</td>
<td>Ventana</td>
<td>TC</td>
<td>TC ≥25%</td>
</tr>
<tr>
<td>73-10</td>
<td>Rabbit</td>
<td>Dako</td>
<td>TC</td>
<td>TC ≥1%</td>
</tr>
</tbody>
</table>

Blueprint phase 2A

22C3, 28-8 and SP263 assays are comparable when used to determine PD-L1 status of patient’s tumour (TPS), SP142 detects less, while 73-10 stains more PD-L1 positive tumour cells


Ab, antibody; NSCLC, non-small cell lung cancer; IHC: immunohistochemistry; TC, tumour cells; IC, immune cells; TPS, tumour proportion score
EGFR
Epidermal Growth Factor Receptor
HOTSPOTS FOR EGFR GENE MUTATIONS

- Type of EGFR alterations: Insertions, deletions, point mutations
- Correlate with: never-smokers, women, adenocarcinoma subtype, Asian ethnicity
- Most common or classical sensitising EGFR mutations:
  - Deletions in exon 19
  - Point mutations in exon 21 (L858R)
- T790M in exon 20 is the most common (~60%) acquired resistance mutation to classical first-second generation TKIs. Sensitivity to 3rd generation TKI

TKI, tyrosine-kinase inhibitor

**EGFR MUTATION ASSAYS**

CAP/IASLC/AMP recommended assays for EGFR genotyping testing

- Non-squamous-cell NSCLC
- Adenocarcinoma
- Large-cell carcinoma
- Other

<table>
<thead>
<tr>
<th>METHOD</th>
<th>TUMOUR DNA REQUIRED (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger direct sequencing</td>
<td>25</td>
</tr>
<tr>
<td>Real time/ TaqMan PCR</td>
<td>10</td>
</tr>
<tr>
<td>High Resolution melting analysis</td>
<td>5-10</td>
</tr>
<tr>
<td>Cobas</td>
<td>5-10</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>5-10</td>
</tr>
<tr>
<td>SNaPshot PCR</td>
<td>1-10</td>
</tr>
<tr>
<td>MALDI-TOF MS-based genotyping</td>
<td>5</td>
</tr>
<tr>
<td>Cycleave PCR</td>
<td>5</td>
</tr>
<tr>
<td>Fragment lenght and RFLP analysis</td>
<td>5</td>
</tr>
<tr>
<td>Allelic specific PCR/ Scorpion ARMS</td>
<td>1</td>
</tr>
<tr>
<td>MassARRAY</td>
<td>1</td>
</tr>
<tr>
<td>PNA- LNA PCR clamp</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing HPLC</td>
<td>1</td>
</tr>
<tr>
<td>Massively parallel/ NGS</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Any appropriate, validated method, subject to external QA

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CAP, College of American Pathologists; IASLC, International Association for the Study of Lung Cancer; AMP, Association for Molecular Pathology; NSCLC, non-small cell lung cancer; NGS, next generation sequencing; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ARMS, amplification refractory mutation system; PNA, peptide nucleic acid; QA, quality assurance; LNA, locked nucleic acid; HPLC, high performance liquid chromatography

Adapted from IASLC Textbook in Thoracic Oncology 2014

EGFR TESTING IN CELL-FREE PLASMA DNA

The dynamic nature of EGFR resistance mechanisms can be monitored in cfDNA

- Insufficient evidence to support the use of cfDNA for the diagnosis of primary lung adenocarcinoma

- When tissue is limited and/or insufficient for molecular testing, physicians may use a cfDNA assay to identify EGFR mutations as an alternative

- Physicians may use cfDNA methods to identify EGFR T790M mutations in lung adenocarcinoma patients with progression or secondary clinical resistance to first-second generation EGFR-TKI

- Testing tissue biopsy is recommended if cfDNA result is negative

Planchard D, et al. Ann Oncol 2018 1;29(Supplement_4):iv192-iv237, © 2018 European Society for Medical Oncology. Published by Elsevier Ltd. All rights reserved.

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ALK
Anaplastic Lymphoma Kinase
**ALK FUSION GENE**

More common: Younger age. Adenocarcinoma subtype

More than 30 partners identified

**Most common partner EML4 (90%)**

Produces oncogenic **EML4-ALK-fusion protein** activation with constitutive kinase activity

Breakpoint within **ALK** always at **exon 20**. Breakpoint within **EML4** variable

**Breakpoints within EML4**

<table>
<thead>
<tr>
<th>Breakpoint</th>
<th>2</th>
<th>6</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>17</th>
<th>18</th>
<th>20</th>
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<tbody>
<tr>
<td>EML4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Most frequent ALK variants**

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Variant</th>
<th>Frequency in ALK+ NSCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EML4 ALK</td>
<td>E13-A20 (V1)</td>
<td>33%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E6a/b-A20 (V3a/b)</td>
<td>29%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E20-A20 (V2)</td>
<td>9%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E14-A20 (V4)</td>
<td>3%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E18-A20 (V5)</td>
<td>2%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E14-A20 (V7)</td>
<td>2%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E2a/b-A20 (V5a/b)</td>
<td>2%</td>
</tr>
<tr>
<td>EML4 ALK</td>
<td>E17-A20 (V8)</td>
<td>1%</td>
</tr>
<tr>
<td>KIF5B ALK</td>
<td>E14-A20 (V7)</td>
<td>2%</td>
</tr>
<tr>
<td>TFG ALK</td>
<td>E18-A20 (V5)</td>
<td>2%</td>
</tr>
<tr>
<td>KLC1 ALK</td>
<td>E14-A20 (V7)</td>
<td>2%</td>
</tr>
<tr>
<td>PTPN3 ALK</td>
<td>E18-A20 (V5)</td>
<td>2%</td>
</tr>
<tr>
<td>STRN ALK</td>
<td>E14-A20 (V7)</td>
<td>2%</td>
</tr>
</tbody>
</table>

Unknown %

More common: Younger age. Adenocarcinoma subtype


NSCLC, non-small cell lung cancer
TESTING FOR ALK GENE FUSION

Any appropriate, validated method, subject to external quality assurance

- FISH is the historical gold standard for testing
- Considered ALK positive if >15% cell with break-apart. The centromeric (green) and telomeric (red) probes flank the ALK locus:
  - Splitting probes of the red and green signals indicates ALK fusion
  - A yellow signal indicates no ALK fusion
- IHC is now becoming the primary therapy-determining test (★): ALK IHC (D5F3, Ventana or 5A4, Novocastra) may be used as a screening test
- NGS is an emerging technology, RNA testing is preferred

BAP, break-apart probe; IHC: immunohistochemistry; FISH: fluorescent in situ hybridization; FFPE, formalin-fixed paraffin embedded


From files of Teixidó C, Reguart N
TESTING FOR ALK GENE FUSION
Diagnostic algorithm that uses IHC as the primary test for ALK identification

- Considered ALK IHC positive if strong granular cytoplasmic staining with/without membrane accentuation
- Occasional cases may be difficult to interpret because of heterogeneous fixation/preservation and/or nonspecific staining artefacts (e.g., staining in alveolar macrophages, neural cells, extracellular mucin, necrosis). In these settings, these cases should also be tested by a validated method (e.g., ALK FISH)

IHC: immunohistochemistry; FISH, fluorescent in situ hybridisation; FFPE, formalin-fixed paraffin embedded
**ROS1 FUSION GENE**

- The **ROS1 fusion gene** appears to be a distinct NSCLC molecular subset susceptible to targeted inhibition.

- Tends to be mutually exclusive with *EGFR, KRAS, ALK* alterations.

- More common:
  - Never- or light smokers
  - Younger age
  - Adenocarcinoma subtype

- The mechanism by which the **ROS1** fusion protein is activated remains unclear.

---

**Many partners, most common CD74 (30%)**

**Produces oncogenic **ROS1** kinase protein**

**Unknown** mechanism of protein activation (no coiled-coil dimerization domain provided)

Breakpoint sites vary **exons 32, 34, 35**

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Breakpoints within **ROS1**

- Breakpoints: 32, 34, 35

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NSCLC, non-small cell lung cancer
TESTING FOR ROS1 GENE FUSION

- **FISH is the trial-validated standard**
  - The ROS1 FISH testing should be performed with a break-apart probe design given the multiple fusion partners described.
  - Considered ROS1 positive if >15% cell with break-apart or with 3’ (green) isolated signals.

- **ROS1 IHC** (D4D6, Cell Signaling Technology or SP384, Ventana) may be used as a screening approach; however, positive ROS1 IHC results should be confirmed by an orthogonal method - cytogenetic method (FISH) or molecular.

- **NGS** is an emerging technology.

---

FISH BAP Menarini

IHC D4D6 Cell Signaling Technology

From files of Teixidó C, Reguart N


*BAP, break-apart probe; IHC: immunohistochemistry; FISH, fluorescent in situ hybridisation;*
BRAF

B-raf Proto-Oncogene
Mutations at BRAF include V600 and no-V600

**BRAF V600** (class I) mutations appear to be a distinct NSCLC molecular subset susceptible to targeted inhibition: BRAF/MEK inhibitors

Typically, mutually exclusive of other oncogenic drivers

It is appropriate to include BRAF as either part of larger testing panels performed initially or when routine EGFR, ALK, and ROS1 testing are negative

**BRAF** testing: any appropriate, validated method, subject to external quality assurance

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**HOTSPOTS FOR BRAF GENE MUTATIONS**


Updated version published 15 September 2020 by the ESMO Guidelines Committee.

N, N-terminal; C, C-terminal; RBD, RAS-binding domain; PL, P-loop; αC, alpha C hélix motif; DF, dimerization interface; CL, catalytic loop; AS, activation site; aa, amino acid

From files of Teixidó C, Reguart N
NTRK

Neurotrophic Tyrosine Kinase
**NTRK FUSION GENE**

- Family of neurotrophic receptors: *NTRK1, NTRK2, NTRK3*
- Transmembrane proteins: *TRKA, TRKB and TRKC*
- *NTRK* fusion gene appears to be a distinct NSCLC molecular subset susceptible to targeted inhibition
- Tends to be mutually exclusive with other oncogenic drivers
- Prevalence in unselected population
  - Occurs across age and smoking status
  - No gender preference

**Breakpoints within NTRK1**

- Breakpoints 12, 14

**NTRK1: The most common fusion in lung**

- Most common partners: *CD74* and *MPRIP*
- Oncogenic constitutive activation of *TRKA*
- Breakpoint sites vary exons 12, 14

**NSCLC, non-small cell lung cancer**

TESTING FOR NTRK GENE FUSION

- Screening for *NTRK* rearrangements may use IHC or NGS, with appropriate testing follow-up to validate a positive result.
- TRK protein testing can be considered as part of broad immunohistochemistry testing.
- A TRK IHC positive test should then be confirmed by a molecular method (FISH, NGS).
- FISH dual colour break-apart probes are easier-to-read than fusion probes in FFPE samples.
- NGS is an emerging technology, RNA testing is preferred.

---

**BAP, break-apart probe; IHC, immunohistochemistry; FFPE, formalin-fixed paraffin embedded; FISH, fluorescent in situ hybridization; NGS, next generation sequencing**

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- Other actionable predictive biomarkers: \textit{RET}, \textit{MET}, \textit{KRAS}, \textit{HER2}, \textit{EGFRins20}
- NGS
- Summary / Conclusions
RET
REarranged during Transfection
RET FUSION GENE

- The **RET fusion gene** appears to be a distinct NSCLC molecular subset susceptible to targeted inhibition.
- Tends to be mutually exclusive with other oncogenic drivers.
- More common:
  - Younger age
  - Adenocarcinoma subtype
- **Selpercatinib (LOXO-292) and pralsetinib (BLU-667)** are selective RET inhibitors approved by EMA for the treatment of NSCLC patients with RET fusions [III, B; ESMO-MCBS v1.1 score: 3]

Six partners, most common **KIF5B** (30%)

- Produces oncogenic **RET** kinase protein
- Protein activation by partner-provided **coiled-coil dimerization domain**
- Breakpoint sites vary exons 11, 12

Breakpoints within **RET**

Breakpoints 11 12

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https://www.esmo.org/guidelines/esmo-mcbs/esmo-mcbs-scorecards

EMA, European Medicines Agency; GoR, grade of recommendation (A-E); LoE, Level of evidence (I-V); NSCLC, non-small cell lung cancer
TESTING FOR RET GENE FUSION

- Currently, testing of RET is generally limited to academic institutions or clinical trial settings.

- FISH is the trial-standard technique (⭐) for RET

- The RET FISH testing should be performed with a break-apart probe design given the multiple fusion partners described

- Considered RET positive if >15% cell with break-apart

- Currently there is no standard (CE-IVD) IHC ab kit for RET fusion detection

- NGS is an emerging technology, RNA testing is preferred


BAP, break-apart probe; IHC: immunohistochemistry; FISH, fluorescent in situ hybridization;
MET

MET Proto-Oncogene
TESTING FOR MET GENE AMPLIFICATION

- Currently, testing of MET is generally limited to academic institutions or clinical trial settings.
- May use any appropriate, validated method, subject to external QA.
- FISH is the trial-standard technique (★), but there is no guideline for cut-off of MET positivity.
- High polysomy occurs when there are multiple copies of chromosome 7 (CEP7) in tumour cells (>5).
- The definition of MET positivity based on gene copy-number has not yet been reached. MET amplification may be classified by using MET:CEP7 Ratio as low (≥ 1.8 to 2.2 ≤), intermediate (>2.2 to <5), and high (≥ 5) (University of Colorado scoring).
- Low-intermediate levels can occur synchronously with other oncogenic mutations and gene rearrangements up to 63% of lung carcinomas.


From files of Teixido C, Reguart N
TESTING FOR MET EXON 14 SKIPPING MUTATIONS

- May be identified with any appropriate, validated method, subject to external QA
- MET\text{\textcopyright14} mutations exhibit a highly diverse sequence composition (insertions, deletions, SNV)
- Targeted NGS-based assays interrogating MET\text{\textcopyright14} as part of a wider gene panel are preferred for screening purposes; better identified using RNA as opposed to DNA sequencing
- Tepotinib and capmatinib are selective MET inhibitors with encouraging activity in NSCLC with MET\text{\textcopyright14}

IAM, immunoglobulin-like, plexins, transcription factor; PSI, plexin–semaphorin–integrin domain; SNV, single nucleotide variation; WT, wild type

From files of Teixido C, Reguart N
KRAS

Kirsten Rat Sarcoma Virus
 More common: white, history of cigarette smoking

Never smokers more likely to have KRAS G12A mutations

Former or current smokers more likely to report KRAS G12T or G12C mutations

Currently, testing of KRAS, is generally limited to academic institutions or clinical trial settings.

Mutations may be identified with any appropriate, validated method, subject to external QA

If available, multiplex platforms (NGS) for molecular testing are preferable

Sotorasib (AMG510), is a selective KRAS G12C inhibitor approved by EMA for the treatment of advanced NSCLC with KRAS G12C mutations [III, A; ESMO-MCBS v1.1 score: 3]
HER2

Human epidermal growth factor receptor 2
TESTING FOR HER2 MUTATIONS

- Correlate with: never-, former-smokers, women, adenocarcinoma subtype, Asian ethnicity

- **Exon 20 insertions**, located at the kinase domain, are the most common HER2 alteration

- Currently, testing of HER2 is generally limited to academic institutions or clinical trial settings.

- Mutations may be identified with any appropriate, validated method, subject to external QA

- If available, multiplex platforms (NGS) for molecular testing are preferable

- **Trastuzumab deruxtecan**, **poziotinib** and **TAS6417** have shown promising activity in NSCLC patients harbouring HER2 mutations

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*NGS, next generation sequencing; NSCLC, non-small cell lung cancer; QA, quality assurance*

EGFR EXON 20 INSERTIONS
Exon 20ins represent ~1–2% of all NSCLC and ~12% of EGFR positive. Resistant to classical EGFR-TK inhibitors

Most frequent EGFR uncommon mutations include G719X (exon 18), S768I (exon 20) and L861Q (exon 21)

Currently, testing of Exon 20ins is generally limited to academic institutions or clinical trial settings

There are two main ways to identify EGFR mutations:

- PCR is typically performed sequentially in order to identify only the most common genomic alterations. Limited to detect molecularly heterogenous mutations, including EGFR Exon 20ins mutations

- NGS is a valuable method to detect the presence of NSCLC biomarkers, including EGFR Exon 20ins mutations, as they cover all the EGFR Exon 20ins

Amivantamab, a potent novel EGFR/c-MET bispecific antibody, is the first targeted therapy approved by EMA (December 2021) for the treatment of EGFR Exon 20ins mutation positive NSCLC

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OUTLINE

- Precision medicine in advanced NSCLC
- Updated ESMO Guidelines for NSCLC genomic testing (2020)
- Optimal Diagnostic Workup of lung cancer:
  - Sample Journey
  - Morphology & Histology
  - Type of samples for Genomic Evaluation
  - Input requirements & Quality Control for Biomarker Testing
- Guidelines recommendations: PD-L1, $EGFR$, $ALK$, $ROS1$, $BRAF$, $NTRK$
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- NGS
- Summary / Conclusions
NGS TESTING

Advantages of NGS testing vs. Single-Marker Molecular Test

<table>
<thead>
<tr>
<th>Single-Marker Molecular test</th>
<th>Multiple-Marker Molecular test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower cost</td>
<td>Lower cost per analysed gene</td>
</tr>
<tr>
<td>(only if 1-2 genes are analysed)</td>
<td></td>
</tr>
<tr>
<td>Short TAT (~3 h)</td>
<td>Long TAT (~3-4 days)</td>
</tr>
<tr>
<td>Greater amount of tissue / DNA if multiple analyses are performed</td>
<td>Less amount of tissue / DNA per gene analysed</td>
</tr>
<tr>
<td>Detects a limited number of known alterations</td>
<td>Allows to detect multiple and unknown alterations</td>
</tr>
</tbody>
</table>

- NGS identifies multiple biomarkers simultaneously without loss of sensitivity and specificity.
- NGS can sequence a whole genome or exome, transcriptomic RNA, or panels of a few to several hundred regions of exons or, to a lesser extent, introns.
- NGS does not address biomarkers that require measurement of protein expression (e.g. PD-L1).
- Can be performed from tumour tissue and ctDNA (liquid biopsy).

Given the high number of currently actionable driver mutations with approved treatments in Europe (EGFR, ALK, ROS1, NTRK, BRAF), and other evolving biomarkers (KRAS, MET, RET, ERBB2/HER2, NRG1), expanded NGS testing panel at diagnosis (rather than 8–12 different tests) could be the most efficient way of identifying optimal therapeutic approaches while avoiding unnecessary re-biopsies.


NSCLC, non-small cell lung cancer; NGS, next generation sequencing; TAT, Turnaround Time
The ESMO Translational Research and Precision Medicine Working Group has established a classification system for molecular aberrations based on the available evidence, supporting their value as clinical targets.

- ESMO Scale of Clinical Actionability for molecular Targets (ESCAT) defines six levels of clinical evidence for molecular targets according to the implications for patient management.

- NSCLC is among the solid tumours with the highest number of ESCAT level I alterations.
ESMO Recommendations for the use of NGS in Lung Cancer

- In non-squamous NSCLC, it is recommended that a tumour (or plasma) sample is profiled using NGS technology, in order to detect all level I alterations.
- There is no current indication for tumour multigene NGS in squamous cell lung cancer.
- Larger NGS multigene panels could be used if they add acceptable extra cost compared with small panels.
- Considering the high frequency of fusions, RNA-based NGS, or DNA-based NGS designed to capture such fusions, are the preferred options.
- It is recommended that hospitals, that run drug development programmes and clinical trials, run multigene sequencing in the context of molecular screening programmes, since lung cancer presents some level II-IV alterations.

The ESMO Precision Medicine Working Group

### ESMO Recommendations for the use of NGS in Lung Cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration</th>
<th>Prevalence</th>
<th>ESCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESCAT TIER EVIDENCE I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>Common mutations (Del19, L858R)</td>
<td>15% (~50% Asian)</td>
<td>IA</td>
</tr>
<tr>
<td></td>
<td>Acquired T790M exon 20</td>
<td>60% of EGFR mutant</td>
<td>IA</td>
</tr>
<tr>
<td></td>
<td>Uncommon (G719X exon 18, L861Q exon 21, S768I exon 20)</td>
<td>10%</td>
<td>IB</td>
</tr>
<tr>
<td><strong>ALK</strong></td>
<td>Fusions (mutations as mechanism of resistance)</td>
<td>5%</td>
<td>IA</td>
</tr>
<tr>
<td><strong>MET</strong></td>
<td>Mutations ex 14 skipping</td>
<td>3%</td>
<td>IB</td>
</tr>
<tr>
<td><strong>BRAF</strong></td>
<td>Mutations</td>
<td>2%</td>
<td>IB</td>
</tr>
<tr>
<td><strong>ROS1</strong></td>
<td>Fusions (mutations as mechanism of resistance)</td>
<td>1-2%</td>
<td>IB</td>
</tr>
<tr>
<td><strong>NTRK</strong></td>
<td>Fusions</td>
<td>0.23-3%</td>
<td>IC</td>
</tr>
<tr>
<td><strong>RET</strong></td>
<td>Fusions</td>
<td>1-2%</td>
<td>IC</td>
</tr>
</tbody>
</table>

**ESCAT TIER EVIDENCE II-III**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration</th>
<th>Prevalence</th>
<th>ESCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KRAS</strong>&lt;sup&gt;G12C&lt;/sup&gt;</td>
<td>Mutations</td>
<td>12%</td>
<td>IIB</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>Exon 20 insertion</td>
<td>2%</td>
<td>IIB</td>
</tr>
<tr>
<td><strong>ERBB2</strong></td>
<td>Hotspot mutations and Amplifications</td>
<td>2-5%</td>
<td>IIB</td>
</tr>
<tr>
<td><strong>MET</strong></td>
<td>Focal amplifications (acquired resistance on EGFR TKI)</td>
<td>3%</td>
<td>IIB</td>
</tr>
<tr>
<td><strong>BRCA 1/2</strong></td>
<td>Mutations</td>
<td>1-2%</td>
<td>IIA</td>
</tr>
<tr>
<td><strong>PIK3CA</strong></td>
<td>Hotspot mutations</td>
<td>1.2-7%</td>
<td>IIA</td>
</tr>
<tr>
<td><strong>NRG1</strong></td>
<td>Fusions</td>
<td>1.7%</td>
<td>IIIB</td>
</tr>
</tbody>
</table>

NGS, next generation sequencing

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Mosele F., et al. Ann Oncol. 2020;31(11):1491-1505 © 2020 European Society for Medical Oncology. Published by Elsevier Ltd. All rights reserved.
OTHER BIOMARKERS OF INCREASING IMPORTANCE IN NSCLC

Co-occurring Gene Alterations

• Co-occurring genomic alterations are emerging as core determinants of the molecular and clinical heterogeneity of oncogene-driven lung cancer subgroups

• Their prevalence and functional importance varies according to the oncogenic driver mutation, clinical context, previous therapeutic exposure and clonal or subclonal nature of the mutation

❖ Co-occurring genomic events in oncogenic drivers are common (KRAS 53% [most common co-occurring event TP53, STK11, KEAP1/NFE2L2], EGFR 50% [most common co-occurring event TP53, RAS-MAPK pathway, PIK3CA], METex14 86% [most common co-occurring event TP53, EGFR, RAS-MAPK pathway])

❖ Concurrent driver oncogenes can be found in ~1-8% of NSCLC patients (commonly involving METex14, KRAS and EGFR mutations)

• Thus, the identification of co-occurring events using NGS (tissue or plasma) is becoming increasingly important as they can be involved in primary and acquired resistance to targeted therapy and immunotherapy
ROLE OF NGS TO IDENTIFY DRUG RESISTANCES

- Almost all patients with NSCLC who benefit from TKIs will eventually develop clinical resistance
- Molecular mechanisms of resistance to TKIs are complex and heterogeneous
- Circulating tumour DNA or tissue-testing (re-biopsy) via broad molecular profiling (NGS) should be considered at the time of progression in oncogenic-driven tumours to identify genomic resistance mechanisms and to guide second- or third-line therapies
- Longitudinal biomarker testing with plasma DNA can provide insights into tumour evolution and heterogeneity during the course of the disease

NSCLC, non-small cell lung cancer; NGS, next generation sequencing

NGS CAN GUIDE OTHER THERAPIES

- Much work is underway to identify alternatives, or more likely, additional biomarkers beyond PD-L1 to enrich patient populations for response to immunotherapy
- NGS might provide additional relevant genomic clues as to which patients may benefit from certain immunotherapies
- These biomarkers are not recommended by guidelines for routine testing in NSCLC

<table>
<thead>
<tr>
<th>Microsatellite instability (MSI)</th>
<th>Tumour Mutational Burden (TMB)</th>
<th>Genomic Alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC → MMR deficient (or proficient) tumour. MSH-2/ MSH6/ PMS2/ MLH1 IHC antibodies</td>
<td>TMB is an emerging immune biomarker in solid tumours</td>
<td>By acting on STING, STK11 modifies the immune microenvironment of the tumour, leading to an immunologically ignored tumour and escape of immune surveillance¹,²</td>
</tr>
<tr>
<td>PCR → MSI (or MSS) tumour</td>
<td>TMB measure the total number of somatic mutations</td>
<td>Several studies have reported the association between alterations in STK11 and lack of benefit from ICI³–⁵</td>
</tr>
<tr>
<td>NGS → MSI (or MSS) tumour</td>
<td>Various measures of tumour mutational burden (TMB) have been explored</td>
<td>STK11 can be detected by comprehensive NGS multigene panels</td>
</tr>
<tr>
<td></td>
<td>An international effort is ongoing to define a consensus on how TMB should be measured</td>
<td></td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; PCR, polymerase chain reaction; MMR, Missmatch repair; MSI, microsatellite instability; MSS, microsatellite stability

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◆ NGS
◆ Summary / Conclusions
All patients with unresectable NSCLC require screening of biomarkers at diagnosis (results within 5–10 days) for selection of first-line therapy.

Current predictive biomarkers recommended by ESMO guidelines include: **EGFR, ALK, ROS, BRAF, NTRK** and PD-L1.

The landscape for other emerging biomarkers is rapidly evolving (**KRAS, MET, RET, HER2**).

The adoption of next-generation sequencing (NGS) into routine practice is more efficient and facilitates the comprehensive characterisation of predictive and emerging drivers, co-occurring genomic events as well genomic alterations driving resistances.

In the era of precision therapy, all professionals involved in lung cancer diagnosis/management should develop their own multidisciplinary tissue management strategy to optimise tissue processing and facilitate reflex testing.
THANK YOU!

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