Objectives:

- Understand the role for liquid biopsy in lung cancer diagnosis and management
- Compare the performance characteristics of existing testing modalities
- Understand the limitations of existing liquid biopsy modalities in clinical care

Targeting EGFR in lung cancer

Baseline 3m 24m

EGFR p.746_750delELREA

Erlotinib therapy

Acquired resistance (median 9-16 mos)

Yu et al. Clin Cancer Res 2013

3rd Gen. TKIs for relapsed EGFR-mutated lung cancer

Best Percentage Change in Target-Lesion Size.

Osimertinib therapy

Why is liquid biopsy the right choice in EGFR-mutated lung cancer?

- Two hot spot mutations (L858R and ex19del) comprise the >90% of activating mutations
- Makes for straightforward assay design
- Tumors with these alterations are responsive to EGFR tyrosine kinase inhibitors

- Majority (~60%) of patients relapse due to acquired T790M mutation
- Also makes for easy assay design
- Tumors with this resistance mutation are response to third generation EGFR TKI (osimertinib) and use of these drugs requires demonstration of the T790M mutation

***REPEAT BIOPSY REQUIRED***
FDA-approved Plasma cfDNA EGFR mutation tests

**At Diagnosis:**
- Roche cobas EGFR Mutation Test v2 for ex19del and L858R
- Approved for use as a companion diagnostic for patients with metastatic NSCLC who are not candidates for biopsy
- ENSURE study
  - 76% of tissue + patients also had plasma +
  - 98% of tissue neg patients were plasma -
- If negative, follow with routine biopsy and EGFR testing on FFPE tissue

**At Relapse:**
- Roche cobas EGFR Mutation Test v2 for T790M
- Approved for use in patients with metastatic NSCLC who are not candidates for rebiopsy
- Efficacy of osimertinib has not been established for patients with plasma+ T790M testing only
- If negative, follow with biopsy and EGFR T790M testing on FFPE tissue

**Approaches to “Liquid Biopsy”**

**Droplet digital PCR (ddPCR)**

Digital PCR:
- Limiting dilutions
- Target DNA is partitioned into multiple replicate reactions
- Some “partitions” contain template and some do not
- End point PCR
  - Yields a binary readout for each partition (0/1)
- Poisson statistics
  - # of template molecules calculated from % of positive reactions
- # of “partitions” determines the dynamic range and the precision of digital PCR

**ddPCR Workflow and Performance**

- Linear over >4 orders of magnitude
- Precision drops at low DNA concentrations

**Clinical Validation of ddPCR for cfDNA EGFR and KRAS mutation detection**

- 10 cc Purple top (EDTA) tube of blood
- Plasma cfDNA isolation
- ddPCR for EGFR ex19del, L858R, T790M and KRAS G12X
- Clinical genotyping
- Results comparison

**BW/DFHCC Clinical Validation of ddPCR for cfDNA EGFR and KRAS mutation detection**

- Phase 1: Validation of the technology in the context of a consented clinical protocol
- Phase 2: Focused clinical assay validation and implementation in a CLIA-certified molecular diagnostics laboratory
Assay Methodology

- Dedicated workflow established to spin blood samples for plasma isolation within 2 hours of collection
  - ≥3ml of plasma used for DNA isolation
- DNA isolation carried out using QIAmp circulating nucleic acid kit (QIAGEN) and vacuum manifold
- ddPCR performed using custom primers/probes on a BioRad platform

Phase 1: ddPCR for EGFR and KRAS hotspots

**Clinical performance characteristics I**

<table>
<thead>
<tr>
<th>EGFR Variant</th>
<th>Accuracy</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity</th>
<th>Reproducibility</th>
<th>LOD (events)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L858R</td>
<td>95%</td>
<td>0.74 (0.22-0.99)</td>
<td>1.0</td>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>Ex19del</td>
<td>90%</td>
<td>0.92 (0.60-0.99)</td>
<td>1.0</td>
<td>95%</td>
<td>3</td>
</tr>
<tr>
<td>T790M</td>
<td>95%</td>
<td>0.97 (0.59-0.99)</td>
<td>1.0</td>
<td>97%</td>
<td>3</td>
</tr>
</tbody>
</table>

Phase 2: CLIA lab validation of plasma cfDNA testing by ddPCR in 23 patient samples

- Advanced NSCLC in need of EGFR genotyping (at relapse and diagnosis)
- Plasma genotyping
  - Plasma EGFR+
  - Biopsy EGFR+
- Additional samples for genotyping
  - Plasma EGFR+
  - Biopsy EGFR+
- Test tumor for other mutations or start chemo

Implementation in clinical practice
**Plasma EGFR testing at relapse**

- Known EGFR-mutated NSCLC at relapse (n=28)
  - Initiate osimertinib (n=4)
  - Plasma EGFR- (n=7)
  - Follow-up biopsy (n=3)
  - Initiate osimertinib
  - Biopsy T790M+ (n=1)
  - Biopsy T790M- (n=2)
  - L858R or ex19del+ T790M+ (n=4)
  - L858R or ex19del+ T790M- (n=3)
  - Repeat testing + initiate osimertinib (n=1)

- Plasma EGFR- (n=5)
  - Initiate chemo

>95% specimen adequacy

75% of samples had detectable EGFR mutation

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**Plasma EGFR testing at “diagnosis”**

- Advanced NSCLC in need of EGFR genotyping (n=27)
  - Plasma-L858R or ex19del+ (n=4)
  - Plasma-EGFR- (n=14)
  - Biopsy EGFR+ (n=3)
  - Other diagnosis (n=7)

EGFR mutations detected in 15% of diagnostic specimens

- Alterations detected in tissue:
  - Mutations analyzed:
    - KRAS
    - EGFR
    - ALK
    - BRAF
    - ERBB2
    - NRAS
    - ROS1
    - RET
    - AKT1
    - MAP2K1
    - HRAS
  - Other/unknown driver (29.9)

- EGFR WT/other mutation (n=7)
- Insufficient/no genomics (n=7)
- EGFR mutations detected in 15% of “diagnostic” specimens.
- Alterations detected in tissue:
  - EGFR G719A/S768I
  - BRAF V600E
  - ERBB2 ex20ins
  - RAS gene mutations

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**Single gene assays for plasma testing: are they good enough in today’s practice?**

**ddPCR**

- Pros:
  - Rapid, specific and quantitative
  - Low cost
- Cons:
  - Limited clinical sensitivity
  - A negative result provides limited information
  - Difficult to multiplex
  - Difficult to detect CNVs and structural variants

**ddPCR: The spectrum of genetic alterations in lung adenocarcinoma**

- KRAS 33.8%
- EGFR 19.1%
- ALK 3.9%
- BRAF 3.8%
- MET 3.0%
- PIK3CA 2.9%
- ERBB2 2.5%
- NRAS 1.0%
- ROS1 1.1%
- RET 1.0%
- AKT1 0.6%
- MAP2K1 0.3%
- HRAS 0.1%

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**Plasma cfDNA NGS for lung cancer**

- cfDNA represents 0.1-5% of cfDNA in stage IV lung cancer
- Advances in NGS design and technology allow for ultrasensitive mutation detection

**Modified amplicon sequencing**

- Preamplification steps
- Molecular barcoding
- TAm-seq (Forshew et al. Sci Trans Med 2012) – 5.6 Kb coverage, MTC >3000, to 0.18% AF

**Modifications to hybrid capture technology**

- Bait design
- Library prep optimization
- Depth of sequencing
- Bioinformatic error suppression
- Commercial options

**CAPP-Seq: Biopsy-free cancer screening**

  - 85% sensitivity and 96% specificity overall for NSCLC mutation detection (ct DNA detection index)
  - Higher sensitivity when stage I patients excluded
  - At a high specificity cutoff of 0.4%, all detected ctDNA mutations classified correctly (No false positives)
**cfDNA mutation detection in early stage NSCLC**

Ohira et al. ddPCR and amplicon NGS (commercial kit) have similar (low; 95% samples positive) sensitivity.

Newman et al: Sensitivity of CAPP-Seq is 50% in stage I-II NSCLC and correlates with tumor volume.

**Single gene assays for plasma testing: are they good enough in today’s practice?**

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**Management of lung cancer resistance requires more sophisticated assays!**

**Complex mechanisms of relapse following third generation EGFR TKI: Use of cfDNA profiling (CAPP-Seq 302Kb/252 gene selector)**

Chabon et al. Nat Comm 2016

**Pre-treatment MET gain predicts inferior survival**

Chabon et al. Nat Comm 2016

60 year old man with relapsed EGFRm lung adenocarcinoma and known T790M mutation, "blowing through osimertinib therapy"

Tissue sequencing:
- EGFR c.2236_2250delGAATTAAGAAGCA (p.E746_A750del), exon 19 – in 77% of 2075 reads
- EGFR c.2369C>T (p.T790M), exon 20 – in 6% of 983 reads

**Perennial lab management questions—now moving into the plasma space**

- Can a targeted NGS panel suit all indications?
  - Mechanism of tumorigenesis (SNV, CNV, SV) varies across organ systems
  - Mutational burden quantification
  - Deep sequencing of plasma using WGS or WES approaches
    - Impractical?
    - Cost prohibitive
Low pass WGS for copy number detection in plasma
Proof of principle in prostate cancer

Plasma ctDNA prescreening to facilitate rational choice of tumor genomic profiling

Take home points:

- *EGFR*-mutated lung adenocarcinoma, with its defined mechanisms of resistance, presents an ideal paradigm for investigation of the clinical utility of plasma cfDNA genomic analysis
- Plasma-based mutation tests should be optimized for specificity, but will only be clinically practical if sensitivity is also acceptable
- Patient characteristics inform the clinical utility of plasma-based testing for diagnostic and predictive uses
  - Number of metastatic sites; overall tumor volume correlate with cfDNA