Liquid Biopsy for Diagnosis and Treatment Monitoring in Esophageal Adenocarcinoma

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Why ctDNA in Esophageal Adenocarcinoma?

General Population

Patients with Reflux Symptoms

Screening Endoscopy

No BE

No Reflux Symptoms

Non-Compliance

Surveillance Endoscopy and Biopsy

80% operable
70% DF at 2 yrs

Progression to EAC

50% operable
8%DF at 2-yrs

Undiagnosed BE

Opportunity for alternative screening approach

Cancer

Opportunity for alternative screening approach
Why ctDNA in Esophageal Adenocarcinoma?

Opportunity: New tool to monitor response to therapy and/or early detection of recurrence

Only 20-40% of patients respond to chemotherapy.

20-30% 50-70%

Chemotherapy
Limited but Promising data on ctDNA in Gastroesophageal Cancer

tcDNA detected in 100% of metastatic cases (n=7)

tcDNA detected at ~10-100 copies/ml of plasma

Bettegowda et al. Sci Transl Med (2014)
Circulating Tumor DNA in EAC

Open Questions:

- How does detection rate and quantity change with tumor stage?
- Does ctDNA quantity change with response to therapy or disease progression/recurrence?

Challenges:

- Need to reliably detect mutations at ~0.05% level.
- Very little DNA in plasma/serum (10-50ng/mL = 1-10 copies of tumor DNA) and it is very fragmented.

Developed modified version of SafeSeq-S using introduction of molecular barcodes ($N_{10-14}$) into NGS libraries using PCR.

Kinde et al., Proc Natl Acad Sci U S A. 2011 Jun 7;108(23)
Barcoding Enables Identification of True Mutations vs Polymerase errors

Wildtype

Wildtype with PCR-induced error

True mutant with PCR-induced error
Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing (SiMSen-Seq)

- Target primers designed with internal hairpin.
  - Protects random barcode sequence during PCR and increases specificity

Stahlberg et al., Nucleic Acids Res. 2016 Jun 20;44(11)
Library Construction is Extremely Quick and Simple

- Two rounds of PCR
- Single purification step.
- Three hours from start to sequence-ready.

Hairpin primers open (~74°C) so adapter primers can amplify off of 1st round primers.
SiMSen-Seq Enables Flexible Multiplexing

![Graphs comparing Single-Plex and 31-Plex](image-url)
Background Consensus Error is Consistently Below 0.1%

12 replicates of 5-plex library covering 417 nucleotides

>90% of all nucleotides displayed consensus read error <0.05% and 99.3% of nucleotides showed consensus error <0.1% with 95% confidence.
Approach

1. Sequence Tumor DNA
2. Isolate Plasma DNA
3. Identify Mutations
4. Barcoded Plasma DNA Sequencing
**Pitt 07: T2N0M0, Stage IB**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Position</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Tumor allele freq</th>
<th>Mutation consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARID1A</td>
<td>Chr1</td>
<td>27107152</td>
<td>G</td>
<td>T</td>
<td>0.2</td>
<td>Nonsense</td>
</tr>
</tbody>
</table>

**Tumor**

**Plasma**

0.15%
### Pitt 29: T2N0M0, Stage IIB

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start Position</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Tumor allele freq</th>
<th>Mutation consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Chr17 7579542</td>
<td>CGTCCGGG</td>
<td>C</td>
<td>0.45</td>
<td>Deletion</td>
</tr>
</tbody>
</table>

#### Tumor

![Graph showing Tumor](image1)

#### Plasma

![Graph showing Plasma](image2)

0.4%
## Pitt 27: T3N2MX, Stage IIIB

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Position</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Tumor allele freq</th>
<th>Mutation consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTNAP5</td>
<td>Chr2</td>
<td>125281996</td>
<td>C</td>
<td>A</td>
<td>0.16</td>
<td>Missense</td>
</tr>
</tbody>
</table>

### Tumor

- **Gene:** CNTNAP5
- **Chr:** Chr2
- **Position:** 125281996
- **Allele 1:** C
- **Allele 2:** A
- **Tumor allele freq:** 0.16
- **Mutation consequence:** Missense

### Plasma

- **Percentage:** 0.2%
### Pitt 25: TxN2M1, Stage IV

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Position</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Tumor allele freq</th>
<th>Mutation consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTNAP5</td>
<td>Chr2</td>
<td>125281996</td>
<td>T</td>
<td>G</td>
<td>0.31</td>
<td>Intron</td>
</tr>
</tbody>
</table>

**Tumor**

```
<table>
<thead>
<tr>
<th>Chromosomal Position</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12525000 - 125281996</td>
<td>0.3</td>
</tr>
</tbody>
</table>
```

**Plasma**

```
<table>
<thead>
<tr>
<th>Chromosomal Position</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12525000 - 125281996</td>
<td>0.012</td>
</tr>
</tbody>
</table>
```
## Circulating Tumor DNA Detection Frequency Increases with Stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total Pts</th>
<th>Pts w/ tumor mutations</th>
<th>Plasma Sequenced</th>
<th>Plasma mutations detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>16</td>
<td>12</td>
<td>6 (50%)</td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>4 (80%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
<td><strong>37</strong></td>
<td><strong>26</strong></td>
<td><strong>14 (54%)</strong></td>
</tr>
</tbody>
</table>
Mutant Allele Frequency in Plasma Increases with Tumor Stage
Improving Detection Sensitivity

• Evolution of SiMSen-Seq:
  • Shorter amplicon sizes
# Amplicons <80bp Give Greater Sensitivity

<table>
<thead>
<tr>
<th>Stage</th>
<th>Pts with tumor mutations</th>
<th>Plasma Sequenced</th>
<th>Plasma mutations detected</th>
<th>Long Amplicons Only</th>
<th>Short Amplicons Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>4</td>
<td>1 (25%)</td>
<td>0/1 (0%)</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>5</td>
<td>3 (60%)</td>
<td>0/1 (0%)</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>III</td>
<td>16</td>
<td>12</td>
<td>6 (50%)</td>
<td>3/7 (43%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>5</td>
<td>4 (80%)</td>
<td>0/1 (0%)</td>
<td>4/4 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>26</td>
<td>14 (54%)</td>
<td>3/10 (30%)</td>
<td>11/16 (69%)</td>
</tr>
</tbody>
</table>
Improving Detection Sensitivity

• Evolution of SiMSen-Seq:
  • Shorter amplicon sizes
  • PAGE purified hairpin/adapter primers in first round PCR
PAGE Purified Primers give Cleaner Libraries

BRAF

NRAS

Non-PAGE

PAGE
Improving Detection Sensitivity

• Evolution of SiMSen-Seq:
  • Shorter amplicon sizes
  • PAGE purified hairpin/adapter primers in first round PCR
    • Higher on-target reads and higher consensus depth
  • True Hi-fidelity polymerase in first round PCR
    • Phusion polymerase reduces background error to <0.02%
Does ctDNA quantity change with Disease Status?

Stage IIIA patient

Point Mutations in: ARID1A, TP53
ctDNA Level Correlates with Clinical Disease Burden

Clinical course:
- Neoadjuvant chemo/radiation
- Esophagectomy
- Palliative radiation
- Progressive Disease
- Death

Tumor allele frequency in plasma:
- TP53
- ARID1A

Days after diagnosis:
0 30 60 90 120 150 180 210 240 270 300 330 360
SiMSen-Seq: Strengths and Weaknesses

• Strengths
  • Easy, fast library construction and relatively simple bioinformatics
    • Implement in individual research and clinical labs
  • Low DNA input requirement (<5ng)
  • Flexible library content
    • Most useful for 1-1000bp coverage
    • Content can be customized easily (individual patient panels, cancer-specific panels, therapeutic panels, companion diagnostics etc.)
  • Low cost for NGS approach (only sequencing regions of interest)
  • Fits perfectly between dPCR and large-scale NGS approaches.

• Weaknesses
  • Up-front assay development needed
  • Limited content relative to other NGS approaches
  • Sensitivity not quite as good as reported for some approaches
ctDNA as a Biomarker in Esophageal Adenocarcinoma

• Early Detection
  • Unclear if sensitivity will be high enough for stage I/II disease
    • But: High risk group known and current paradigm is failing

• Treatment response and recurrence monitoring
  • May have value for rapid identification of response to therapy
  • May identify pre-clinical recurrence
  • May identify residual disease following “curative” treatment

• Other
  • Prognostic biomarker in stage I/II disease
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