Next Generation Sequencing for Solid Tumors Diagnostics: Current Practice and New Developments

Larissa V. Furtado, MD FASCP
Speaker Disclosure

In the past 12 months, we have not had a significant financial interest or other relationship with the manufacturer(s) of the product(s) or provider(s) of the service(s) that will be discussed in our presentation.

Larissa V. Furtado, MD FASCP
Program Objectives

1. Demonstrate familiarity with next-generation sequencing (NGS) and the various applications for which it can be used in the oncology setting.

2. Recognize the indications, specimen requirements, assay design considerations and limitations of NGS-based testing for solid tumors.

3. Understand interpretive principles for review and reporting of clinically relevant findings within the proper solid tumor contexts.

Sections

1. Introduction to Personalized Oncology Diagnostics
2. Technology, Test Selection and Test Capabilities
3. Future Trends in Solid Tumor Genomic Diagnostics
Personalized Medicine in Oncology

Prediction

Diagnosis

Prognosis

Therapy
Inborn genetics:
- Genetic disease
- Risk factors

Disease Genetics:
- Diagnosis
- Prognosis
- Therapy

Disease Genetics:
- Early screening

Disease Genetics:
- Residual disease testing
- Resistance mutation surveillance
Cancer Genomics Targets

**Mutations (TS and OG)**
- Point mutations
- Insertions and deletions (indels)

**Structural Variations**
- Large scale deletions/duplications
- Fusions/rearrangements
- Aneuploidy
- Chromothripsis

**Epigenetics**
- Altered DNA methylation
- Altered histone methylation
- Altered DNA-protein interactions
- Altered chromatin structure

**Gene Expression**
- OG or TS dysregulation
- Pathway activation
- MicroRNAs
- LncRNAs
- Alternative Splicing
- Allele-specific expression
- RNA binding protein interactions
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NGS – Effective at All Size Scales

- **PCR-based Methods**
- **CGH/SNP Arrays**
- **Fluorescence In Situ Hybridization (FISH)**
- **Cytogenetics**
- **MLPA**
- **Southern Blotting**
- **Next Generation Sequencing (NGS)**

**DNA Replication Defects**
- Point Mutation
- Small Insertion/Deletion
- Larger Duplication/Deletion
- Trisomy/Monosomy
- Altered Ploidy

Base Pairs (log scale)
Lung Cancer Targets

Point Mutations
EGFR L858R, G719S, etc.
KRAS
PIK3CA
BRAF

Small Deletions
EGFR exon 19

Copy Number Alterations
MET amplification
EGFR amplification

Gene Fusions
ALK (e.g. EML4-ALK)
RET
ROS1
NTRK1

Lung Adenocarcinoma
NGS vs. Traditional Methods

• Multiple anomalies at different genomic scales can be assayed simultaneously.
• More sensitive than Sanger sequencing.
• Single extraction and single test instead of multiple tests.
  – Cost effective
  – Improved turn-around time by avoiding sequential testing
  – Tissue preservation – many genes simultaneously assessed from single extraction
• Potential for discovery of novel actionable targets.
• Extreme flexibility of analysis types.
NGS Oncology Challenges

- Cost of implementation
- Significant requirement for informatics infrastructure and expertise
- Rapidly changing nature of technologies
- No standardized guidelines available for data analysis, interpretation and reporting
- Uncertainty of reimbursement
- Uncertainty of clinical utility
- Intense market competition
Commercial Testing Landscape

• Assay types
  – Cancer profiling panels (small to large):
    • Mutations
    • Copy number changes
    • Translocations
  – Circulating tumor DNA assays
  – Immune clonality profiling
    • Lymphoma (including residual disease testing)
    • Tumor-associated lymphocytes

• Hype vs. reality?
A significant number of mutated genes have been identified in the four major tumor types, although only a limited set have been shown to be "driver" mutations.

The number of actionable mutations remains limited

- Pharma companies are developing drugs against a number of other gene targets as well as 2\textsuperscript{nd}- or 3\textsuperscript{rd}-line treatments
TAGRISSO™ (AZD9291) approved by the US FDA for patients with EGFR T790M mutation-positive metastatic non-small-cell lung cancer

Objective response rate of 59% and duration of response of 12.4 months.
Opportunity for Panel Testing – lung cancer example

<table>
<thead>
<tr>
<th>Molecular techniques</th>
<th>Estimated current test cost(^1)</th>
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<th>NGS(^2)</th>
<th>Total</th>
<th>Lung screen</th>
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<tr>
<td>Manufacturer share</td>
<td>$2,300-2,800</td>
<td>$2,000-3,500</td>
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<tr>
<td>Service provider share</td>
<td>$1,600-2,400</td>
<td>$800-1,600</td>
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</tbody>
</table>

\(^1\) Manufacturer share \& Service provider share

\(^2\) NGS = Next-Generation Sequencing
Sections

1. Introduction to Personalized Oncology Diagnostics
2. Technology, Test Selection and Test Capabilities
3. Future Trends in Solid Tumor Genomic Diagnostics
ABCs of NGS
Next Generation Sequencing

Random DNA
Next Generation Sequencing
Next Generation Sequencing

ACTGGTCAGCT

TCTCTCTCATAT

GCTAAAATAAAA

CCCCCATTTATAA

TCTCTCTCATAT

TTCAATATCGGG

GCTAAAATAAAA

TTCAATATCGGG
Library Preparation

Sample Genomic DNA

5' -> Sample Genomic DNA -> 3'
Sample DNA
(Fragments < 1kb)
Library Preparation

Sample DNA
(Fragments < 1kb)

Adaptor

Adaptor
Library Preparation

Barcode Sequence
Sample DNA (Fragments < 1kb)
Barcode Sequence

Adaptor

Adaptor
Sequencing
Sequencing
Sequencing
Data: a list of sequences of DNA molecules sampled from the input library
Informatics

Data
(List of Sequences)

?

Biological Result
Informatics (Alignment)

READ SEQUENCE
GACTTGACCAGCAGTAGTATACGCGATCTGG

...AACGTGCATTAGCCGACTTGACCAGCAGTAGTATACGCGATCTGGAGACTAGACCTGCAACC...

Chromosome Sequence

Chromosome and position assignment
(e.g. chr 2, position 123,224,414)
Informatics (Alignment)

Variant Location

GACTTGACCACGCACTGATATACGCGATCTGG
...AACGTGCATTTAGCCGACTTGACCACGCACTGATATACGCGATCTGGAGACTAGACCTGCAACC...

Chromosome Sequence

Chromosome and position assignment
Indel Alignment

READ SEQUENCE
ACGTGCATTTAGC TGGAGACTAGACCTGC

...AACGTGCATTTAGCCGACTTGACCGCAGTAGTATACCGGATCTGGAGACTAGACCTGCAACC...

Chromosome Sequence
Variant Detection

Is it real?
Cancer – Low % Mutations

Tumor cell percentage

Tumor heterogeneity

Low mutation allelic percentage

Increased Depth Improves Mutation Detection

- >99% sensitivity for 50% mutations
- >99% sensitivity for 10% mutations
- >99% sensitivity for 5% mutations

Estimated Sensitivity vs. Sequencing Depth

- 50% mutation
- 10% mutation
- 5% mutation
- 1% mutation

Foundation Medicine, 2011
Agilent Users Meeting, Boston, MA
How to select targets for sequencing?
Tissue

Prepare Unselected Fragments

Prepare Selected Fragments

Whole Genome Sequencing

Targeted Sequencing

Blood
Targeted Sequencing – Hybrid Capture

1. Genomic DNA → Construct shotgun library → Fragments → Hybridization → Pulldown → Captured DNA
2. DNA sequencing → Mapping, alignment, variant calling
Whole Genome Sequencing

Targeted Capture-Based Sequencing

Increased depth for improved low% variant detection
Targeted Sequencing – Amplicon Assays
Simplified Assay Type Comparisons

Amplicon Systems
- Low DNA Input
- Turnaround Time
- Broad Coverage
- Small/Medium Indels (<100 bp)
- Copy Number Alterations
- Structural Alterations

Hybrid Capture
Amplicon Assays for Minute Specimens
ARUP – Solid Tumor Mutation Panel

~10 ng FFPE DNA, 10-15% tumor cells:
EGFR mutation negative
KRAS c.34G>T, p.G12C (NM_033360) – 5% MAF

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<thead>
<tr>
<th>Gene</th>
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<td>EZH2</td>
<td>JAK3</td>
<td>PTEN</td>
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<td>FBXW7</td>
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<td>ALK</td>
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<td>FGFR3</td>
<td>KRAS</td>
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<td>SRC</td>
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<td>GNAQ</td>
<td>NOTCH1</td>
<td>STK11</td>
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<td>HNF1A</td>
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<td>TP53</td>
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<td>HRAS</td>
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<td>PIK3CA</td>
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<tr>
<td>ERBB4</td>
<td>JAK2</td>
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</tbody>
</table>
Low Input Allows for Direct Testing of Cyto Smears
Capture Assay Flexibility

Mutations/Indels
- EGFR Exon 19del

Rearrangements
- TP53 11kb deletion

Copy Number Events
- EGFR/MET/other amplification

Gene Fusions
- KIF5B-RET Fusion
Need to ensure good sampling!
But...don’t be fooled!

BAD Sampling

Library Prep & Sequencing
Cancer – Difficult Specimens

Formalin Fixation

Fragmented, nicked, crosslinked, end-damaged DNA

Scant Tissue

Low yield of DNA

Brain photo: Gaetan Lee
Macrodissection – Laboratory Method to Enrich Tumor Content

Pathologist reviews H&E for adequate tumor cell content
Selects and marks best tumor area

Key Considerations:
- Total yield
- Tumor cell %

Corresponding area marked on serial unstained slide

Tumor area lifted from slide for DNA/RNA extraction

Slide courtesy from Bryan Betz, PhD
As a lab, how do you think about planning an assay?

- What size (# genes)?
- What type of preparation?

- There is no clear consensus in this field about what is the ideal test.
Assay Design Considerations

Small Panel | Large Panel | Exome | Genome
---|---|---|---
Cost per sample
Validation Time/Cost
Value for Discovery
What’s the right size assay?

Smaller Targeted Assays

- Some clinicians
- Cancer specimens
- Validation effort
- Cost
- Reimbursement

Larger Comprehensive Assays

- Most clinicians
- Clinical requirements
- Translational research
- Lab Competition
- Technology
Summary: NGS Assay Development

• NGS allows many types of anomalies in many genes simultaneously.

• Design and strategy decisions are complex.
  – Many contributing factors and influences.
  – Many assay type choices with different pros and cons.
After Data Analysis…

- The back-end challenges of clinical NGS implementation can be daunting!
  - Proper databasing of clinical variants.
  - Workflow for analyzing individual cancer cases:
    - How many people involved?
    - Handing off responsibility and ensuring proper review.
    - Confirmatory assays for variants as necessary.
  - Generation of appropriate reports for clinicians.
  - Integration with electronic medical records and hospital information systems.
Cancer NGS Interpretation

Variants

Inherited Variants

Somatic Mutations
Cancer NGS Interpretation

- Variants
  - Inherited Variants
  - Somatic Mutations
Cancer NGS Interpretation

- Variants
  - Inherited Variants
  - Somatic Mutations
    - “Driver” Mutations
    - Bystander Mutations
Cancer NGS Interpretation

- **Variants**
  - Inherited Variants
  - Somatic Mutations
    - "Driver" Mutations
    - Bystander Mutations
VARIANT:
chr4 55593464 A>C

Gene: KIT
Exonic missense
c.1621A>C, p.M541L

Protein Effects Prediction
Pathway Analysis
Evolutionary Conservation

Annotation Pipeline

Inherited Variants
1000 Genomes, dbSNP
Exome Variant Server

Cancer Variants
COSMIC db
TCGA db

Lab Variants
Internal db

PublMed
Somatic Variants

- Benign / Not Reported
- Variants of Uncertain Clinical Significance
- Pathogenic Variants

**ARUP Tiers**

<table>
<thead>
<tr>
<th>Tier</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tier 1 – Actionable</td>
<td>(FDA Approved Therapies in Patient Tumor Type, Established Diagnostic or Prognostic Significance)</td>
</tr>
<tr>
<td>Tier 2 – Potentially Actionable</td>
<td>(FDA Approved Therapies in another Tumor Type, Potential Diagnostic or Prognostic Significance)</td>
</tr>
<tr>
<td>Tier 3 – Variants of Unknown Significance (VUS)</td>
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</tbody>
</table>
### Pending Samples

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<tr>
<th>Accession</th>
<th>Test Mnemonic</th>
<th>Test DTA</th>
<th>Remaining TAT</th>
<th>Current Stage Task</th>
<th>In-Lab Status (Millennium)</th>
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<td>16238113106</td>
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<td>0 Days, 20Hours</td>
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Showing 1 to 3 of 3 entries
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<th>Nuc. Change</th>
<th>Prostate Change</th>
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<th>1KG Freq</th>
<th>ESP Frequency</th>
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<th>ARUP Obs.</th>
<th>dbSNP Id</th>
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<th>Interpretation</th>
<th>Classification</th>
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Patient Information

Patient Name: 
Sex: 
Date of Birth: 
Ordering Physician: 
Clinical History: Metastatic melanoma 
Sample Source: Liver

Generate Report: 16-238-113106 (SOLID NGS)

Overall Result: See Note

Choose References

Template: Solid Tumor 3 Tier
Use Global Template

Include the following variants:

*Notes shown here are not included in the report:

- IDH NM_059896.3: c.315C>T p.Gly105...
- TP53 NM_000546.5: c.215C>T p.Pro72...
- RET NM_020975.4: c.3712C>T p.Arg1241...
- APC NM_000038.5: c.4479G>A p.Thr1493...

Total Characters (including background): 3169

SAMPLE SOURCE: Liver
CLINICAL HISTORY: Metastatic melanoma

I. TIER 1: Actionable (FDA Approved Therapies in Patient Tumor Type, Established Diagnostic or Prognostic Significance)

NONE DETECTED

II. TIER 2: Potentially Actionable (FDA Approved Therapies in another Tumor Type, Potential Diagnostic or Prognostic Significance)

NONE DETECTED

III. TIER 3: Variants of Unknown Significance (VUS)

1. MET c.2980C>T p.Arg976Cys (R976C) (NM_000245.2)

Interpretation: This variant occurs in the juxtamembrane domain and is recognized in the literature as either Arg976Cys or Arg985Cys. It has been observed infrequently in lung cancer (CCOSAM database), colorectal cancer (Fumagalli et al., 2010), chronic myelomonocytic leukemia, endometrial cancer, thyroid cancer and melanoma (Tyner et al., 2010). The transformation ability of this variant is uncertain as some in vitro studies have shown a mild increase in cell proliferation and transformation (Ma et al., 2003), whereas others show no growth or transformative advantage (Tyner et al., 2010). In vivo studies in mice suggest this variant may increase susceptibility to lung cancer (Zaffaroni et al., 2003). This variant is listed in ClinVar as having conflicting interpretations of pathogenicity (benign, likely benign, and uncertain significance). This variant is listed as a germline variant in dbSNP (rs343059476) with a minor allele frequency (MAF) of 0.001, in the Exome Aggregation Consortium with a MAF of 0.0029, and in the NHLBI Exome Sequencing Project with a MAF of 0.0035. The functional consequence in this context is unknown. The clinical significance, if any, is uncertain.
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Emerging Genomics Targets

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- LncRNAs
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- RNA binding protein interactions

**Other Applications**
- Circulating tumor DNA assays
Circulating Tumor DNA (ctDNA)

Fig. 1. Potential applications of ctDNA.
Clinical Sensitivity Depends on Tumor Type

- Bladder (n=3)
- Colorectal (n=24)
- Gastric-oesophageal (n=17)
- Pancreatic ductal (n=17)
- Breast (n=14)
- Melanoma (n=19)
- Hepatocellular (n=9)
- Head and neck (n=6)
- Neuroblastoma (n=9)
- Medulloblastoma (n=14)
- Prostate (n=5)
- Renal cell carcinoma (n=5)
- Thyroid (n=4)
- Glioma (n=27)

Frequency of cases with detectable ctDNA (%)
ctDNA: a promising marker of recurrence.

Fig. 5. The relationship between ctDNA concentration (mutant fragments per milliliter) and 2-year survival. The association between survival and ctDNA concentration was assessed, holding known prognostic factors (age, ECOG PS, and CEA) constant. The 2-year survival was estimated on the basis of a multivariable Cox regression model, in which ctDNA concentration level was transformed with a natural spline function.
Fig. 6. Heat map of acquired resistance mutations to EGFR blockade in ctDNA from patients with metastatic CRC.
Cell-free DNA technologies: Achieving high sensitivity

Cost/Extent of Testing

- Digital Droplet PCR
  1-5 “hotspot” mutations

- BMF_amplicon
  parts of 10-20 genes

- BMF_capture
  Full exonic coverage of 25-50 genes

Slide courtesy from Sabine Hellwig, PhD
Challenge: Finding Low% Mutations in NGS Data
Molecular Barcode Proof-Reading

Library Amplification

Redundant Sequencing

Kinde I et al. PNAS 2011;108:9530-9535
Molecular Barcode Proof-Reading
Molecular Barcode Proof-Reading

NPM1 c.860_863dup
AF = 0.1%
Absolute count of amplified wildtype and mutant copies
Detected copies/mL plasma or MAF can be calculated
**ARUP Validated ddPCR assays**

**EGFR T790M**

- Resistance mutation causing **loss of sensitivity** to EGFR-targeted primary TKI therapy (erlotinib, gefitinib) in non-small cell lung cancer (NSCLC)
  - Average progression on TKI after 11 months (100% progression rate)
  - T790M accounts for 2/3 of cases with acquired resistance
- Next generation TKI with (prospective) FDA approval:
  - Osimeritinib (*Tagrisso, Astra Zeneca*) – accelerated approval Nov 2015
  - Rocelitinib (*Clovis*) – delayed approval

**BRAF V600E**

- Activating point mutation in the BRAF kinase domain
  - 50% of melanoma, 20-40% of thyroid cancers, 8-15% of colorectal, 1-4% of NSCLC
  - Valine to glutamate accounts for ~90% of mutations at V600
- Associated with increased sensitivity to
  - Dabrafenib (BRAF inhibitor)
  - Vermurafenib (BRAF inhibitor)
  - Trametinib, cobimetinib (MEK inhibitors)

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Slide courtesy from Sabine Hellwig, PhD
T790M Resistance monitoring: Tarceva cohort

NSCLC EGFRmut+

TKI ~ 11 months

Progression EGFR-IR+ T790M

Next gen TKI ?

Progression not yet observed by scan (>2 months)

Detection by ddPCR 2 months before progression
Dx by scan

Detection by ddPCR 5 months before progression

Days on Tarceva
Molecular margins, treatment response, & early recurrence monitoring by ctDNA

Biopsy or Surgery

Targeted and/or Chemotherapy

Recurrence Monitoring

FFPE Solid Tumor NGS

Pre-OP Post-OP Pre-Therapy Intervals During Therapy Post-Therapy

Intervals at time of imaging

Correlate ctDNA data with imaging, whole body and tumor perfusion sampling

Slide courtesy from Sabine Hellwig, PhD
**BRAF V600E - Surgical Margin monitoring**
*(Melanoma case 1)*

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**Pre-surgery**
- cfDNA: 2.1ng/mL plasma
- V600E probe intensity: 55:1170, 4.5%
- WT probe intensity: 31 copies/mL plasma

**48h post surgery**
- cfDNA: 15.3ng/mL plasma
- V600E probe intensity: 10:6421, 0.16%
- WT probe intensity: 5 copies/mL plasma

**14d post surgery**
- cfDNA: 21.7ng/mL plasma
- V600E probe intensity: 4:9076, 0.04%
- WT probe intensity: <LOD

**5w post surgery**
- cfDNA: 5.8ng/mL plasma
- V600E probe intensity: 0:2080

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Slide courtesy from Sabine Hellwig, PhD
BRAF V600E - Melanoma case 2 – MAF v. copies/mL

Inflation in cfDNA content skews MAF!

Slide courtesy from Sabine Hellwig, PhD
Liquid Biopsy: Replacing Tissue?

• Clearly not all tumors shed DNA into the blood in appreciable amounts.
• Discovered mutations do not necessarily come from the tumor of interest.
• Resistance mutations present in a subset of cells may not be discoverable by ctDNA, but may be detectable in tissue.
• Tissue testing seems likely to remain first-line, although there are great possibilities for liquid biopsy for surveillance.
Conclusions

- NGS continues to revolutionize personalized diagnostics in oncology.
- NGS is allowing for comprehensive analysis of difficult specimen types (small biopsies, cytology specimens and plasma and body fluid specimens).
- Many applications are emerging beyond simple sequence analysis (ctDNA, immune profiling, gene expression, epigenetics, etc.).
- Currently, the trends are towards increasing the breadth of analysis for each patient.
- Questions remain about optimal testing strategies.
Thanks! Questions?

- Larissa Furtado – larissa.furtado@hsc.utah.edu
  – larissa.furtado@aruplab.com