Oncogenomics:
From Cancer Genome to Biological Mechanism

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Agenda

- Overview of Oncogenomics
  - Types of DNA biomarkers
  - Types of DNA mutations
- Experimental Strategies for Cancer Genomics
  - Techniques Used
    - Discovery
    - Validation
- qBiomarker Mutation Assay Design and Validation
- Disease-focused Mutational Profiling
  - qBiomarker Somatic Mutation Arrays
- Summary & Questions
Oncogenomics

Synonymous with Cancer Genetics (DNA based research)

Research Focus for Oncogenomics/ Cancer Generics

- Understanding molecular mechanism of transformation, progression and metastasis
  - Monitor alterations in genome

- Improve Diagnosis
  - Use biomarkers for earlier detection of cancer

- Prognosis
  - Use biomarkers to stratify tumors with different outcomes

- Therapeutics
  - Develop targeted therapeutics or determine the most effective treatments
Biomarker for Disease Mechanism
In some cases primary breast cancer tumor cells show no evidence of HER2/neu amplification, but metastatic circulating tumor cells in those patients do show increased expression.

Therapeutics
Drug compounds may behave differently in a similar profile of sample, based on different tumor genotypes
- Ex. 30% of Breast Cancers overexpress HER2.
  - However, not all respond to HER2 therapy (i.e. Herceptin®)
  - This may indicate a different HER2 mutation occurring in samples, thereby yielding different outcomes.
DNA Biomarkers in Oncogenomics

Alterations to the genome that can be detected

Epigenetic Changes
- Changes in DNA Methylation

Chromosomal Amplification or Loss
- Entire Chromosomes

Copy Number Variations (CNV)
- Inherited changes in gene copy number

Copy Number Alterations (CNA)
- Acquire changes in gene copy number

Single Nucleotide Polymorphisms (SNP)
- Inherited nucleotide changes

Somatic Mutations (DNA mutations)
- Acquired mutations not present in all cells
What Kinds of DNA Mutations are There?

- **Point Mutation**
  - A → G

- **Frame Shift Mutation / Insertion / Duplication**
  - ACGTACGTC
  - ACGATAACGTC
  - ACGGATACGTC

- **Deletion**
  - ACGTACGTC
  - ACGTACGTC

- **Inversion**
  - ACGTACGTC
  - ACTGACGTC

*From 1 – 100s of bases*
Oncogenomics Databases

- **COSMIC** is a resource which displays the data generated from the Cancer Genome Project.

- **Cancer Genome Anatomy Project** from National Cancer Institute also has banked much information of research on cancer genome, transcriptome, and proteome.

- **Progenetix** is another oncogenomic reference database, presenting cytogenetic and molecular-cytogenetic tumor data.

- **Oncomine** has compiled data from cancer transcriptome profiles.

- **IntOGen** (Integrative Oncogenomics) integrates human oncogenomic data classified by tissue type using the ICD-O terms.

- **International Cancer Genome Consortium** collects human cancer genome data.

- **HGMD**: The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff

- **dbRBC**: Blood Group Antigen Gene Mutation Database: NCBI

- Animal model such as Retrovirus Tagged Cancer Gene Database (RTCGD) has compiled retroviral and transposon insertional mutagenesis in mouse tumors.
Survey: Which alterations are you studying?

Alterations to the genome that can be detected

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Oncogenomics application: Somatic mutations

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Somatic Mutations (DNA mutations)
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How do DNA Mutations Occur?

• Copying Errors
  • as part of Replication Process
    • Estimated Error Rate of DNA Polymerase: 1 in $10^8$ bases
      • Human Genome = 2.9 billion bases ($2.9 \times 10^9$ bases)

• DNA Damage
  • Resulting from Environmental Agents, such as:
    • Sunlight (UV)
    • Cigarette Smoke
    • Radiation
Current Methods Used for Mutation Detection

Different Methods for Different Experimental Questions

Pre-Screen

Discovery
- SNP Chips
- NGS

Validation
- qPCR
- Sequencing
- Mass Spectroscopy

Diagnostic Test
## Analysis Methods for DNA Mutations

<table>
<thead>
<tr>
<th>Method</th>
<th>Benefits</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point Mutation Analysis</td>
<td>Exact</td>
<td>Time Consuming</td>
</tr>
<tr>
<td>Quantify DNA Targets</td>
<td>Fast</td>
<td>Time Consuming</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Reliable, High-throughput</td>
<td>Need Instrument/Training</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>Exact</td>
<td>Time Consuming</td>
</tr>
<tr>
<td>High Resolution Melt</td>
<td>Inexpensive, Fast, Simple</td>
<td>Base change identity not obvious</td>
</tr>
<tr>
<td>Microarray</td>
<td>Large Coverage</td>
<td>Sensitivity, Protocol complex, Need Instrument/Training</td>
</tr>
<tr>
<td>Next-Generation Sequencing</td>
<td>Exact</td>
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## Comparison of different technologies

<table>
<thead>
<tr>
<th></th>
<th>qPCR</th>
<th>Sequenome OncoCarta *</th>
<th>Sanger Sequencing</th>
<th>NGS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technology</strong></td>
<td>Real-time PCR based:</td>
<td>Multiplex PCR, Extension,</td>
<td>Individual PCR, Clean-up,</td>
<td>Sequence enrichment, library construction,</td>
</tr>
<tr>
<td></td>
<td>-single-step handling</td>
<td>Mass spec:</td>
<td>Extension, CE:</td>
<td>NGS -lengthy procedure</td>
</tr>
<tr>
<td></td>
<td>-close tube</td>
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<td>-high cost for high coverage</td>
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<tr>
<td></td>
<td></td>
<td>-risk of contamination</td>
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<td>Focused content</td>
<td>~300 mutations in 19 genes</td>
<td>Homebrew assay, low gene</td>
<td>Genome wide, however this also depends on</td>
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<td></td>
<td>- Expanding coverage</td>
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<td>coverage, Some levels of</td>
<td>sequencing depth. -Detecting 1% mutation =</td>
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<tr>
<td></td>
<td>- Relevant mutations</td>
<td>mutations</td>
<td>mutation discovery</td>
<td>1000X sequencing depth</td>
</tr>
<tr>
<td></td>
<td>-Customization</td>
<td>-not so easy to customize</td>
<td></td>
<td></td>
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<td><strong>Samples</strong></td>
<td>5~10ng fresh/frozen</td>
<td>500ng fresh or FFPE</td>
<td>Fresh/frozen, FFPE</td>
<td>Several ug of DNA</td>
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<tr>
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<td>200~500ng FFPE</td>
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<td><strong>Sensitivity</strong></td>
<td>1%</td>
<td>10%</td>
<td>&gt;20%</td>
<td>Depends on sequencing depth</td>
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<td><strong>Throughput</strong></td>
<td>High-Throughput based off of</td>
<td>&gt; 96/d</td>
<td>High sample/low content</td>
<td>Depends, long waiting time</td>
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<td></td>
<td>throughput</td>
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<td>Real-time PCR cycler</td>
<td>Sequenome Mass spectrometer</td>
<td>CE sequencer</td>
<td>NGS sequencer -limited access</td>
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<td>-wide accessibility</td>
<td>-limited access</td>
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<td>Simple</td>
<td>Complicated</td>
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Measurement of Sensitivity: (important with heterogeneous samples)

In a population of 100 total cells, sensitivity of:

• qPCR: 1-2%
  • 1-2 mutant DNA + 98-99 wild-type DNA

• Pyromark Sequencing: 5%
  • 5 mutant DNA + 95 wild-type DNA
  • Method of DNA sequencing based on the “sequencing by synthesis principle”

• Sequenom/Microarray: 10%
  • 10 mutant DNA + 90 wild-type DNA
Research Question 1: Does my sample have a particular mutation?

9 FFPE lung cancer samples, 1 placenta FFPE sample
Sample origin: Caucasian (8), Asian (1), mixed sex & smoking status, adenocarcinoma

Is a known EGFR mutation present?

Research Question 2: Does my sample have a mutation in the EGFR pathway?

Screen known mutations in:
AKT  BRAF  EGFR  KRAS  HRAS  NRAS  MEK1  PIK3CA  PTEN
qBiomarker Somatic Mutation Assay Pipeline

Specifications for an assay pipeline

- Specificity
- Sensitivity
- Ease of use
- Integrated control features
- Coverage
qBiomarker Somatic Mutation Assay Pipeline

How were these specifications addressed

- **Specificity**
  - Combine two qPCR methods
  - Every lot of every assay is tested before shipment

- **Sensitivity**
  - Preferentially amplify mutant alleles
  - Threshold Cycle cut-offs incorporated into QC specs

- **Ease of use**
  - Compatible with virtually any qPCR platform
  - Compatible with fresh, frozen or fixed samples
  - Mastermix included and matches your desired instrument

- **Integrated control features**
  - Copy Number Control Assay – what if your gene is deleted

- **Coverage**
  - Over 1000 somatic mutation assays in 96 genes
  - Custom design process available
ARMS: Amplification Refractory Mutation System

- Strategy in which Primers Discriminate Among Templates Differing by a Single Nucleotide

- Oligos with a mismatched 3’-residue **WILL NOT FUNCTION** as Primers in a PCR Reaction

- But with the 3’-residue matching at the exact spot of the mutation
Primer Elongation & Probe Activation with ARMS®

Wildtype template

ARMS primer does not extend on wild type DNA

Common Region

Mutant template

ARMS primer extends on mutant target DNA
ARMS®: A Closer Look

Sample with mutation

\[ \text{ARMS Mutation Primer} \rightarrow \text{TCAGTAAA} \]
\[ \text{Template: Mutation} \rightarrow \text{AGTCAGTT} \]

Mutation Locus:
\[ \text{C is correct for WT} \]

Why this works: **Internal Mismatches: Enzyme Requirement Less Stringent**

Sample without mutation

\[ \text{ARMS Mutation Primer} \rightarrow \text{TCAGTAAA} \]
\[ \text{Template: Wild-Type} \rightarrow \text{AGTCAGTCC} \]

Mutation Locus:
\[ \text{C is correct for WT} \]

Won’t Work

Add’l Mutation: Location Varies

ARMS Mutation
The Exonuclease Activity degrades a hybridized non-extendable DNA probe during the extension step of the PCR.

- DNA Probe: Hybridizes to a region within the amplicon and is dual-labeled with a reporter dye and quenching dye.
Assay qualification criteria

Assays are qualified based on

- **Specificity** (>8 Ct difference between 10ng 100% mutant and wildtype gDNA template) (<0.4% cross-reactivity)

- **Sensitivity** (requires Ct<31 for 10ng DNA)

- **Efficiency** All assays have 100% ± 10% amplification efficiency
Assay detection limit test for qBiomarker Braf V600E assay. A series of 10ng genomic DNA samples, which contain genomic DNA from A375 cell line mixed with WT genomic DNA at different ratios, were tested on qBiomarker Somatic Mutation Assay for Braf V600E with or without whole genome amplification. Mutation detection limit for this assay is determined to be ~1%. 

WGA = Whole Genome Amplification
• ABL1 (14)
• AKT1 (2)
• AKT3 (1)
• ALK (10)
• APC (63)
• ATM (6)
• BRAF (16)
• CBL (4)
• CDH1 (5)
• CDKN2A (19)
• CEBPA (10)
• CRLF2 (1)
• CSF1R (1)
• CTNNB1 (51)
• EGFR (35)
• ERBB2 (15)
• EZH2 (3)
• FBXW7 (7)
• FGFR1 (2)
• FGFR2 (2)
• FGFR3 (12)
• FLT3 (20)
• FOXL2 (1)
• GATA1 (4)
• GATA2 (1)
• GNAQ (3)
• GNAS (6)
• HNF1A (2)
• HRAS (14)
• IDH1 (5)
• IDH2 (4)
• JAK2 (7)
• KIT (32)
• KRAS (24)
• MEK1 (4)
• MET (9)
• MPL (3)
• NF1 (14)
• NOTCH1 (8)
• NOTCH2 (1)
• NPM1 (7)
• NRAS (17)
• PDGFRA (27)
• PIK3CA (59)
• PIK3R1 (3)
• PIK3R5 (1)
• PTCH1 (5)
• PTEN (42)
• PTPN11 (16)
• RB1 (5)
• RET (10)
• RUNX1 (10)
• SMAD4 (1)
• SMARCB1 (7)
• SMO (1)
• SRC (1)
• STK11 (17)
• TET2 (1)
• TP53 (113)
• TSHR (15)
• VHL (16)
• WT1 (4)

Total Entries in COSMIC
105,929

Our >1000 assays cover over 77,819 of these occurrences
Mutation Frequency and Phenotypic Overlap

Heterogeneous disease does not mean random

Various cancers have associated mutations
- Comparable to SNPs

Phenotypes arise from disruption of similar mechanisms or pathways
- Mutation in a tyrosine receptor could be similar to mutation in a target protein

Mutations in the same gene are phenotypically different
- Mutations that inactivate versus activate

Solution: Mutational Profiling based off of sample knowledge or other criteria
Somatic Mutation PCR Array

- Definition: Collection of mutation specific assays for multiple mutations in a single gene, as well as mutations present in genes in downstream signaling activities

- Example: EGFR Pathway Somatic Mutation PCR Array
  - EGFR Mutations: 29 Different mutations present in the EGFR gene
  - AKT1 Mutations: 1
  - BRAF Mutations: 12
  - KRAS Mutations: 18
  - HRAS Mutations: 11
  - NRAS Mutations: 12
  - MEK1 Mutations: 4
  - PI3K Mutations: 7
  - PTEN Mutations: 6
### Pathway-focused Arrays
- EGFR pathway
- ERBB2 (HER-2/neu) pathway
- PDGFR pathway
- FLT3 pathway
- KIT pathway
- MET pathway
- FGFR pathway
- Ras/Raf pathway
- RTK panels (two of them)
- PI3K pathway
- P53-Rb pathways
- β-Catenin/CTNNB1-APC pathway

### Cancer-focused Arrays
- Lung cancer
- Breast cancer
- Colon cancer
- Melanoma
- Hematopoietic neoplasms
- Lymphoid neoplasms
- Skin cancers
- Brain cancers
- Soft Tissue cancers
- Thyroid cancer
- Bladder cancer
- Liver cancer
- Gastric cancer
- Ovarian cancer
- Pancreatic cancer
- Endometrial cancer
- Head and Neck cancer
- Esophageal cancers

### High Content Arrays
- Cancer Comprehensive
- Oncogene
- Oncogene & TSG
- Tumor Suppressor Genes
- Tyrosine Kinase signaling
qBiomarker Somatic Mutation PCR Array Layout

Greek Symbol = Gene
# = Mutation

• For Normalization
• Assays detect non-variable region (not ARMS-based design)
• Designed to control differences in sample input and quality
Profile disease or pathway-focused mutation profiling

1. Isolate genomic DNA from fresh, frozen or FFPE samples using QIAamp or DNeasy kits recommended in the handbook.

2. Add qBiomarker Probe mastermix to genomic DNA.
   - 200 – 500 ng fresh/frozen DNA
   - Less requires WGA
   - 500 – 3000 ng FFPE DNA

3. 1 sample goes on 1 plate

4. Standard 40 cycle PCR on most real-time Thermocyclers.

5. Upload C_T values to Data Analysis Webportal
• Purpose: Qualitative assessment of samples
  • Is the DNA Mutation Present and if so, which one?

• Data Analysis Methods depends on:
  • 1. Availability of wild-type sample
  • 2. Sample type/quality
    • Cell lines
    • Fresh or frozen samples
    • FFPE samples
  • 3. How many samples?

• NOTE: Compare Presence of Same Mutant Assay Across Samples
• NOTE: Each sample harbors only a single or at most few mutations
Data Analysis: Average $C_t$ Method

Recommended for experiments using:

- FFPE samples
- Large #s of samples (> 4)
- Wide range of sample quality
- Wild-type controls not necessary
- Assumes that for a given locus, mutation occurs in small % of samples.

$\therefore C_t^{\text{average}} = \text{Mutation Assay Background in Wild-Type Sample}$

$\Rightarrow$ Compare $C_t^{\text{sample}}$ to $C_t^{\text{average}} = \Delta C_t$

$\Rightarrow$ If $C_t^{\text{sample}} \ll C_t^{\text{average}} \Rightarrow$ Sample has Mutation
FFPE cell line samples (breast cancer & prostate cancer cell lines)

- **MDA-MB-231 as a positive control:**
  - G464V BRAF and G13D KRAS mutation status gathered from COSMIC database and confirmed by utilizing DxS KRAS PCR test and BRAF Pyrosequencing assay

9 FFPE lung cancer samples, 1 placenta FFPE sample

- **Sample origin:** Caucasian (8), Asian (1), mixed sex & smoking status, adenocarcinoma
- **Expected EGFR mutation rate:** ~10%
The two known mutations in MDA-MB-231 cell line FFPE sample were correctly called: 2 Kras and 1 EGFR mutation (~10%) called in 3 lung cancer FFPE samples.
Recommended for experiments using:

• Small #s of samples (< 4)

• Basic Cell Line Experiments….Looking for Mutation in just a couple of cell line samples

• Results affected by sample quality (Wild-type and Test Samples)

• Wild-type controls NECESSARY
  - Wild-type controls MUST be of high-quality

• Considers Copy # Changes
  - NORMALIZES ($\Delta \Delta C_t$)

• Caveats
  - Penalizes good quality samples (False Negatives)
  - Rewards bad quality samples (False Positives)
Data Analysis: $\Delta\Delta C_t$ Method

* A raw $C_t$ cutoff for $C_t$ (sample locus X) will be applied (usually 35); If sample $C_t >$ cutoff $C_t$, the sample will NOT be called “mutant” for that locus.

Normalized $C_t$ For Allele/Mutation Site = $\Delta C_t = C_t^{\text{MUT}} - C_t^{\text{CNA}}$

<table>
<thead>
<tr>
<th></th>
<th>Mutation Assay</th>
<th>CNA</th>
<th>$\Delta C_t$</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>40</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>Mutant</td>
<td>30</td>
<td>27</td>
<td>3</td>
</tr>
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$\Rightarrow \Delta\Delta C_t = 9$
ΔΔC_t Method: Example with Cancer Cell Lines
qBiomarker Copy Number Arrays

Profile disease or pathway-focused copy number profiling

Platform-independent
• Compatible with almost any qPCR instrument
• Choose appropriate mastermix for instrument type

~ 84 mutations tested per array

1 sample per PCR plate/ring

Gene Selection
▪ Arrays by disease
▪ Arrays by pathway
▪ Arrays by functional gene classes
What do you need to detect mutations by qPCR?

**DNA Isolation**
- QIAamp DNA Mini Kit (51304 or 51306)
- DNeasy Blood & Tissue Kit (69504 or 69506)
- QIAamp DNA FFPE Tissue Kit (56404)

**Whole Genome Amplification** (optional)
- Repli-G

**Individual Assays**
- qBiomarker Somatic Mutation PCR Assays
  Includes Mutation and Reference PCR Assay
  Includes Mastermix (based on instrument)

**Mutation Profiling**
- qBiomarker Somatic Mutation PCR Arrays
- Custom qBiomarker Somatic Mutation PCR Arrays
  Includes (based on instrument)

**qBiomarker Data Analysis**
Summary

Validating Oncogenomics

Somatic Mutations are important biological changes with ramifications for human health

Experimental Solutions for Oncogenomics

Discovery experiments
- Looking for new mutations

Validation / Pre-Screen / Hypothesis-driven experiments
- qPCR

Better assays yield reliable sample stratification
- Bench-validated assays with superior performance

Mutation Profiling
- Pathway-focused
- Custom designs