Practical Molecular Testing in Surgical Pathology
Martin P Powers, MD
UCSF Pathology and Laboratory Medicine
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Disclaimer
• I am one of the directors of the Molecular Diagnostics Lab at UCSF
• The lab and medical center may receive revenue from some of the testing hereafter described.

Some assumptions
• Know that DNA, RNA and protein exist
• Know that DNA is transcribed into RNA, which is then translated into protein
• Know that techniques like PCR, DNA sequencing and FISH exist
  – To be explained in more detail later

Outline (approximate)
• Oncogenes and Tumor Suppressors
• Detecting clinically relevant mutations in oncogenes and tumor suppressors
  – Large DNA rearrangements
    • FISH, RT-PCR, LOH, etc
  – Small DNA mutations
    • PCR, sequencing, etc.
• Technical issues
• We can only scratch the surface in 45 min
Introduction to oncogenes and tumor suppressors

Hallmarks of Cancer
- Genes that promote these hallmarks
  - ONCOGENES
  - accelerators
  - Mutations “switch them on”
- Genes that inhibit these hallmarks
  - TUMOR SUPPRESSORS
  - “brakes”
  - Mutations “knock them out”

Cell. 2000 Jan 7;100(1):57-70

Oncogenes and tumor suppressors

• Oncogenes
  - Promote cell division/invasion or Inhibit apoptosis (cell death)
  - Gain of function mutation
  - Dominant mutation
    • Only one allele mutated (usually) to promote tumorigenesis
    • Wild type copy can still be present

Somatic mutations and oncogenes

• Mutation in oncogene is only in tumor cells
  - (sporadic tumors)
• Surrounding stroma, inflammatory and all other cells are wild-type.

Cells of body don’t have mutation
Cells of tumor only have mutation
Somatic mutations and oncogenes

Chromosome with oncogenic mutation

Wild-type (normal) chromosome

Oncogenes

- Mechanisms
  - Amplification
  - Overexpression by translocation
  - Mutation, hyperactivation
    - Translocation
    - Point mutation

- Types of Genes
  - Growth Factors
  - Growth Factor Receptors
  - Signal Transducers
  - Transcription factors
  - Cyclins and Cyclin dependant kinases
  - Anti-apoptotic molecules

Oncogenes

Growth factors, signal transduction and the cell cycle

Growth factors, signal transduction and the cell cycle

Tumor suppressors

- Tumor suppressors
  - Gatekeepers (direct)
    - Inhibit cell division
    - Promote apoptosis
  - Caretakers
    - Protect the genome (DNA repair)
    - Indirect: Increase mutations in oncogenes and gatekeepers

Tumor suppressors
Tumor suppressors

- Mechanisms
  - Point mutations
  - Deletions
  - Silencing of gene expression (or combo for each copy)
- Types of genes
  - Inhibitory growth receptors/ligands
  - Pro-apoptotic molecules
  - Cell adhesion molecules
  - Inhibitors of signal transduction
  - Cell cycle inhibitors
  - Sensors or enzymes for DNA repair

Tumor suppressor genes

- Must be inactive to promote tumorigenesis (lose your brakes)
- Recessive mutations
- Must lose both copies in the tumor
  - Knudson's two-hit hypothesis
  - Inherit the first mutation in inherited cancer syndromes
- Often one of the copies is lost through a “loss of heterozygosity” whereby the entire chromosome, or chromosome arm, or large deletion deletes one of the copies, while a point mutation (or other small mutation) was on the other copy.
Somatic vs inherited genetics (for tumor suppressor genes)

- **Inherited**
  - Rb mutation
  - All cells of body inherit one mutation
  - Predisposition to RB = familial retinoblastoma
    - Dominant inheritance

- **Somatic (sporadic)**
  - Some cells get second hit (somatic)
  - Retinoblastoma tumor
  - Two Mutations acquired in postnatal life in same cell
  - Two mutations required for tumor
    - Cellular recessive

Detecting clinically relevant oncogene mutations

Amplification

- **Examples**
  - ERBB2 (HER2/neu)
    - Worse prognosis in breast cancer
    - Candidate for anti-ERBB2 therapy (such as trastuzumab) for breast, GU, stomach/GE cancer
  - EGFR
    - Worse prognosis and diagnostic for GBM
  - MYC (or MYCN)
    - Worse prognosis in neuroblastoma and medulloblastoma

Mechanism of oncogene activation: amplification
HER2(ERBB2) fish

- 17q11.2-12
- Often amplifies a homogenously staining region
- FISH
  - HER2 = orange
  - CEP17 = green

HER2(ERBB2) fish

- 17q11.2-12
- Often amplifies as a homogenously staining region
- FISH
  - HER2 = orange
  - CEP17 = green
- HER2/CEP17 ratio
  - <1.8 = not amplified
  - >2.2 = amplified
  - 1.8-2.2 = equivocal

Example photograph: ERBB2

Positive for Amplification

Negative for Amplification

Example photograph: EGFR

Positive for Amplification

Negative for Amplification
Over-expression by translocation

- Common in lymphomas
  - IGH-BCL2
    - Follicular lymphoma
  - IGH-MYC
    - Burkitt lymphoma
  - IGH-CCND1
    - Mantle cell lymphoma

Translocation to create a fusion gene

- BCR-ABL
  - CML and AML
- EWSR1-FLI1
  - Ewing Sarcoma
- NPM-ALK
  - Anaplastic large cell lymphoma
- EML4-ALK
  - Non-small cell lung cancer

Mechanisms of oncogene activation: translocation to active chromatin

Mechanisms of oncogene activation: translocation - novel chimeric gene
FISH for translocations

- **Breakapart**
  - (one partner of translocation)

FISH for translocations

- **Fusion**
  - (both partners partner of translocation)
FISH advantages

• Visualize tumor cells separate from normal cells
• Small tumor samples can be studied
• Visualize any heterogeneity in the tumor

PCR for translocations

• Over-expression translocations
  – No fusion transcript
  – Must do DNA PCR
    • Sensitivity depends on spacing of breakpoints
• Fusion translocations
  – Fusion transcript
  – Allows for RT-PCR

What is PCR

• Exponential amplification of DNA based on sequence specific primers
  – Amplify specific sequence between the primers
• If you start with RNA
  – RT-PCR
  – RT (Reverse Transcription) step first converts RNA to cDNA and then you amplify like PCR
PCR = exponential target amplification

- Final copies = $n^2$
- $C = \text{cycles}$
- $N = \text{starting number of copies}$
- If $n = 1$
  - After 1 cycle = 2, after 2 cycles = 4, after 3 cycles = 8, after 4 cycles = 16, after 5 cycles = 32
  - After 10 cycles, 1024
  - After 20 cycles, ~1 million
  - After 30 cycles, ~1 billion

Translocations on DNA

- Need the breakpoints to be close together
- IGH-BCL2
- 70% sensitive

Translocations on RNA

- Advantage of exon specific primers
- BCR-ABL

***Adapted from Genes Chromosomes Cancer. 2001 Oct;32(2):97-111***

Activating point mutations in oncogenes

- KRAS
  - Resistance to EGFR therapy in colorectal carcinoma
- BRAF
  - Resistance to EGFR therapy (colon), candidate for BRAF therapy (melanoma)
- EGFR
  - Response to EGFR therapy in lung cancer

How many mutations are there

- COSMIC
  - Catalogue of somatic mutations in cancer
  - Database of literature reported mutations in cancer in different genes
  - [http://www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/)

BRAF in COSMIC
KRAS in COSMIC

EGFR in lung
- Mutations mostly in 4 different exons (18-21)
- 2 hotspots
- L858 point mutation (exon 21)
- Indels near amino acid 746-747 (exon 19)

Techniques for mutation detection
- Sequencing
- Allele specific PCR
- Real-time PCR
- Allele specific extension (post-PCR)
- others

References:
Issues to consider for mutation testing

- Analytical sensitivity
  - How much DNA is needed?
    - Total amount of DNA, and therefore specimen required
    - 1 ng of DNA represents about 330 diploid cells
    - 1 diploid genome is about 6.6 pg of DNA
  - How many % mutant copies need to be present to detect a mutant signal?

% mutant burden

- DNA Sequencing
- Analytic Sensitivity ~20%

To dissect or not to dissect

12 tumor cells, 8 lymphocytes: 30% mutant allele burden

To dissect or not to dissect

12 tumor cells, 8 lymphocytes, 50 fibroblasts and endothelial cells: 8.5% mutant burden, but can dissect a discreet tumor mass with less normal cells
To dissect or not to dissect

12 tumor cells, 8 lymphocytes, 50 fibroblasts and endothelial cells: 8.5% mutant burden, but no discreet tumor mass

Important things to think about
• Analytical sensitivity and whether or not your lab dissects
• Choice of mutations that will be tested for: is the panel comprehensive enough for your clinicians need

Testing for tumor suppressors
• DNA sequencing
• Loss of functions mutations
• May be anywhere in gene

Effects of Fixative on DNA
• Buffered formalin: damages DNA, but not as bad as unbuffered formalin
• Alcohol based fixatives: good preservation of DNA
• Decalcification: strong acids are very bad for DNA

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**Test for loss of other copy of gene**

- Deletion/Absence of other copy by FISH
- LOH of polymorphic markers in neighborhood of gene

**Deletion testing by FISH: 1p/19q**

- Good prognosis and prediction of chemotherapeutic response
- Actual tumor suppressors not cloned yet
- Tend to delete the entire 1p and 19q chromosome arms
LOH studies

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<th>Normal</th>
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<td>4.5</td>
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<tr>
<td>D1S162</td>
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To dissect or not to dissect

12 tumor cells, 8 lymphocytes, 50 fibroblasts and endothelial cells: 8.5% mutant burden, but no discreet tumor mass

Testing for tumor suppressor loss: Testing a phenotype: MSI

- Microsatellite instability
  - A molecular phenotype
  - Results from genetic lesions in the MMR machinery (mismatch repair)
  - Inherited mutations in MMR
    - Lynch, Turcot, Muir-Torre Syndromes
  - Sporadic loss of MMR
    - Sporadic MSI-high tumors

Microsatellite instability

- Microsatellites
  - Oligonucleotide repeats
    - Single nucleotide, dinucleotide, trinucleotide, etc
  - Slippage during replication
  - Repaired by Mismatch repair machinery
  - Absence of MMR - unstable
Mismatch repair

- “Caretakers”
- MSH6 and MSH2
- MLH1 and PMS2
- Excise and repair mismatches and looped out bases in DNA

In the figure below, patients 1 and 3 show instability

Lynch vs. Sporadic MSI-high tumors

- 15% of colorectal carcinoma: MSI-high
- Lynch (3-5%) and Sporadic (10-12%)
- Sporadic
  - MLH1 hypermethylation (CIMP-high)
    - Sometimes seen in Lynch (as a mechanism of LOH)
  - BRAF mutations
    - Mutated in sporadic (~50%)
    - Wt in lynch (almost always)
Thank you for listening

Any Questions?

References
