Molecular Pathology for the Anatomic Pathologist

Jennifer L. Hunt, M.D.
Cleveland Clinic
Course Director

• Jennifer Hunt, M.D.
  – ENT and Endocrine surgical pathologist
  – Director of Molecular Anatomic Pathology
Audience

• Anatomic Pathologists
• Pathology Residents
• Non-molecular biologists
Handouts

• Syllabus
• CD of course
• Listing of labs
Goals

- Basics of molecular assays
- Application of molecular testing to AP specimens
- Select cases that would benefit from molecular testing
Techniques

- Microdissection
- Loss of Heterozygosity
- DNA Fingerprinting
- Translocation analysis (RT-PCR)
- Microsatellite instability testing
Case Discussions

- **Neoplasia**
  - Case 1 (8:35 – 9:05)
  - Case 2 (9:10 – 9:35)

- **Identity testing**
  - Case 3 (9:40 – 10:10)

- **Break (10:10 – 10:35)**
  - Case 4 (10:35 – 11:00)

- **Prognostics and therapeutics**
  - Case 4 (11:00 – 11:25)
  - Case 5 (11:25 – 11:45)

- **Questions and Answers (11:45 – 12:00)**
Introduction to Molecular Biology

- DNA
- RNA
Case One
Case 1

- Techniques illustrated
  - Loss of heterozygosity analysis
  - Assessing clonality
Case 1

- **75 Year old Male**
  - 30 pack-years of smoking
  - Alcohol

- **1997: Squamous cell carcinoma of Larynx**
  - Laryngectomy, bilateral necks
  - Stage: T3 N1 MX
  - Post-op radiation
Case 1

- Followed clinically
- 2002: Solitary lung mass
  - Lobectomy
  - Mediastinal lymph node sampling
Case 1

Question:
Is the lung tumor a metastasis or a second primary tumor?
Case 1

- **Metastasis**
  - Short time interval
  - Same histology
  - Multiple lesions
  - *No in situ* component

- **Second primary**
  - Long interval
  - Different histology
  - Single lesion
  - *In situ* component
Clonality Assessment

• Molecular approach to identifying related clones
Case 1
HUMARA

- Androgen receptor
  - Human Androgen Receptor Assay
- X-inactivation patterns
HUMARA Non-Clonal
HUMARA Clonal
HUMARA

- Gender (Female only)
- Age related changes
- Patch size
Tumor Genetic Fingerprint

- Permanent DNA damage
- Unique mutations
  - Tumor specific
Genetic Mutations

- Deletions
- Amplification
- Translocations
- Point Mutations
- Chromosomal numeric differences
  - Ploidy
DNA Fingerprint

- Genotype
- Fingerprint
- Mutational profile
DNA Fingerprint

- Germline genetics (person)
  - Identity Testing
- Somatic mutational profile (tumor)
  - Genotyping
Tumor Suppressor Genes

- Most common tumor mutations
- Accumulate over time
- Found in all types of neoplasia
- Measure deletion (loss of heterozygosity or allelic imbalance)
  - Assume second hit
Knudson’s Hypothesis
Tumor Suppressor Gene Analysis

- Requirement
  - Discrimination of the two copies of the gene
Vocabulary

• DNA polymorphisms
• Short tandem repeats (STRs)
  – Microsatellites
• Variable nucleotide tandem repeats (VNTRs)
• Single nucleotide polymorphisms (SNPs)
DNA polymorphisms

Genetically Informative?
DNA Polymorphisms

• **Short tandem repeats**
  
  – 2 to 7 basepairs in length
    
    • Dinucleotide, Trinucleotide, Tetranucleotide…

  
  – Repeated a variable number of times

  ATCG ATCG ATCG ATCG ATCG ATCG
DNA Polymorphisms

- Allelic frequency
  - Population dependent
- High variability is desirable
DNA Polymorphisms

INFORMATIVE
DNA Polymorphisms

NON-INFORMATIVE
Tumor suppressor gene
Genetic changes

- Tumors
  - Expand clonally
  - Accumulate tumor suppressor gene mutations

- DYNAMIC PROCESS!
Case 1

• Mutational profiles
  – Compare different tumor foci
  – Compare tumor at different times
  – Measure clonal expansion and instability
Microdissection

DNA extraction

Polymerase chain reaction
PCR product detection

• Gel: Agarose or polyacrylamide
• Capillary electrophoresis
• Quantitative PCR
  – Taqman
  – LightCycler
Agarose Gel Electrophoresis
Capillary Electrophoresis
Capillary Gel Electrophoresis
Loss of Heterozygosity

Normal Cell → Cancer Cell

Heterozygous → Homozygous
PCR Analysis

Normal

Loss of heterozygosity
Capillary electrophoresis

Normal

Tumor 1

Tumor 2

R=1.09

R=3.6

R=19
Interpreting LOH

\[
\frac{\text{Tumor allele 1}}{\text{Tumor allele 2}} = \frac{\text{Normal allele 1}}{\text{Normal allele 2}}
\]

Normal = 0.70 to 1.43
Capillary electrophoresis

Normal

Tumor 1

Tumor 2

$\frac{1.09}{3.6} = 0.30$

$\frac{1.09}{19} = 0.06$
Case 1

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Genotyping Comparison

- Completely matched or unmatched
Case 1

• DIAGNOSIS:

• Primary laryngeal carcinoma
• Lung metastasis
Genotyping Comparison

- Tumors are dynamic
  - Accumulate additional mutations
  - Not sampling all clones
Combined Matched & Unmatched

- Visual inspection
- Calculated likelihood ratio
  - Mathematical model

\[ (n, m_1, m_2, p) = \binom{n}{m_2} p^{2m_2} \times \binom{n - m_2}{m_1} x 2^{m_1} x p^{m_1} x (1 - p)^{2n-2m_2-m_1} x 0.5^{m_2} \]

\[ (n, m_1, m_2, p_1, p_2) = \sum_{i=1}^{m_2} \binom{n}{i} p_1^i (1 - p_1)^{n-i} x \text{PIO} (n - i, m_2 - i, m_1, p_2) \]
Case 1

- **Visual inspection**

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- **Likelihood ratio**
  - LR = 1298
  - High probability of relationship
Tumor Genotyping

- Other Applications
  - Relating metastasis with primary tumor
  - Collision tumor analysis
  - Pre and post-therapy analysis
Case Two
Case 2

• Techniques illustrated
  – RT-PCR
  – Gene Sequencing
  – Fractional allelic loss (FAL)
Case 2

- 55 Year old male
  - Symptomatic goiter
  - Multiple large nodules
  - Total thyroidectomy
Case 2

- Thyroidectomy
- Surgeon concerned: Adherent, fibrotic, large
- Gross: Three encapsulated nodules
- Frozen section and touch preps
Frozen Section

• Deferred:
  – Hurthle cell lesions
Case 2

Questions:
1. Intrathyroidal metastasis?
2. Widely invasive vs. minimally invasive?
3. Prognosis?
Oncogenes

Proto-oncogene  Oncogene

Normal Cell  Cancer Cell
Tumor Genes

- **Tumor suppressor genes**
  - Recessive
  - Inactivation

- **Oncogenes**
  - Dominant
  - Activation
Oncogene Example

mRNA
Oncogene Mutation Detection

- **Protein level**
  - Immunohistochemistry
- **mRNA level**
  - RT-PCR
- **DNA level**
  - PCR
  - Sequencing
Oncogenes

- Point mutations
- Amplifications
- Translocations
Sarcoma Translocations

- Synovial sarcoma
- Alveolar rhabdomyosarcoma
- Ewing’s Sarcoma
- Clear cell sarcoma
- Desmoplastic small round cell tumor
- Myxoid/round cell liposarcoma
- Extraskeletal myxoid chondrosarcoma
Detecting translocations

- Chromosomal level (cytogenetics)
  - Fresh tissue available?
- Genomic level (PCR, FISH)
  - Are breakpoints clustered?
- mRNA level (RT-PCR)
  - Optimal preservation?
- Expression level (IHC)
  - Is protein expression specific?
Classical Cytogenetics

- Fresh cells capable of dividing
- Visualize whole chromosomes
  - Large deletions
  - Copy number changes
  - Translocations
Genomic Level—PCR

- Breakpoints must be clustered
  - Breakpoint cluster regions
- Example
  - Follicular Lymphoma
Translocations

DNA

1000’s of basepairs
Genomic Level—FISH

- Translocations
- Copy number
  - Specific chromosomes or genes
mRNA Level—RT-PCR

- Translocation assay of choice
  - Tissue preservation is critical
mRNA
Protein Level—IHC

- **Expression of protein product**
  - Is it specific?
  - Is it sensitive?
Thyroid Carcinoma

• RET-PTC: Papillary carcinomas
• PAX8-PPARγ: Follicular carcinomas
PPARγ-PAX8

PPARγ (3p25)

PAX8 (2q12)

PAX8-PPARγ
RET translocations

H4
Exon 1

RET
Exon 12 - 20

ELE1
Exons 1-5

RET
Exon 12 - 20
RET-PTC

- Many different variations
- No prognostic value
- Most common: RET-PTC1
RET-PTC

- FVPTCa: 35%
- PTCa: 25%
- FCa: 15%
- FA: 5%
PAX8-PPARγ translocations

PAX8
Exons 1-7, 8, or 9

PPARγ
Exons 1-7
PAX8-PPARγ

• Prognostic significance
  – Unclear

• Expression profile
  – Suggests different categories
PAX8-PPAR_γ

![Bar graph showing the percentage distribution of FVPTCa, PTCa, FCa, and FA. The FCa bars are significantly higher than the others.](image-url)
Thyroid Translocation Analysis

- Breakpoints not clustered
- Protein expression not specific

FISH
RT-PCR
Handling

- **Tissue for translocation analysis**
  - *Fresh tissue* → Cytogenetics
    - Handle quickly
    - Send directly
  - *Snap frozen tissue* → DNA/RNA
    - Handle quickly
    - Store at -70 C
Handling—Paraffin Tissues

• Fixation times 12-24 hours
  – Avoid over or under-fixing
• Block storage: Cool area (don’t melt)
• Slides: Cut fresh for the assay
RT-PCR

- Template mRNA
- Reverse Transcriptase
- Primers for RT

\[ \text{cDNA} \]

PCR Reaction
RNA Extraction

- Rapid handling
- Cold Temperatures
- Avoid RNAse
RNA From Paraffin
RNA From Paraffin
Primers for RT

- **Random hexamers**
  - Anneals randomly

- **Oligo dT**
  - Anneals to polyA tail of mRNA

- **Product specific primers**
  - Anneals to location of interest
RT Primers

mRNA

AAAAAAA

TTTTTT
Reverse Transcription

mRNA

RT

A
RT-PCR from Paraffin

- Use of random hexamer primers
- Small PCR product size essential
- Robust controls important
- Interpretation of negative results
RT-PCR
Sample Gel

Lane 1: DNA Ladder
Lane 2: Water Control
Lane 3: Positive Control
Lane 4: Negative Control
Lane 5: Sample
Lane 6: RNA Control
Southern Blot Confirmation

RT-PCR Product

Southern Blot Hybridization

Confirmation Of translocation
Sequencing Confirmation

RT-PCR Product

Sequence PCR product

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Sequencing
Molecular Genotyping

• Panel of tumor suppressor genes
  – Fractional allelic loss
    • Mutation rate and instability
    • Clonal expansion
  – Correlates with malignancy
  – Correlates with outcome
Genotyping

- Non-aggressive tumors
  - Low fractional allelic loss

- Aggressive tumors
  - High fractional allelic loss
Fractional Loss

- WIFCa: 80%
- MIFCa: 60%
- FA: 40%
Anaplastic Transformation
Fractional Allelic Loss

Anaplastic: 70%
WIFCa: 50%
PTCa: 30%
Case 2—Results

|   | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | p | q | r | s | t |
| T1|   |   |   |   |   |   |   |   |   |   |   |   |   | X |   |   |   |   |   |   |   |
| T2|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | X |   |   |   |   |   |

FAL = 58%

Likelihood Ratio = 1243
Case 2

Final Diagnosis

Widely invasive Hürthle cell carcinoma with intrathyroidal spread
Other RT-PCR Applications

- **Minimal Residual Disease**
  - Detect rare tumor cells
  - Requirement
    - Known positive disease specific mutation

- **Micrometastatic disease**
  - Experimental
    - Melanoma (Tyrosinase, Melan-A)
    - Breast carcinoma (CEA, cytokeratin)
    - Esophageal carcinoma (Cytokeratin)
Other Genotyping Applications

- Parathyroid neoplasia
- Gliomas
- Squamous dysplasia
- Pancreatic neoplasia
Break
Case Three
Case 3

• Techniques illustrated
  – Microdissection
  – DNA fingerprinting
  – PCR
Case 3

• 36 year old woman
  – Vague breast mass on exam
  – Mammogram negative
  – Underwent FNA in surgeon’s office
Case 3

• FNA Biopsy
  – Highly cellular aspirate
  – Diagnostic for carcinoma
Case 3

- **Clinical impression:** Benign changes

- **Surgery**
  - Conservative lumpectomy
Case 3

- **Lumpectomy**
  - Benign fibrous breast tissue
  - FNA biopsy site seen
  - No evidence of malignancy (submitted in toto)
Case 3

- Pathologist concerned
  - FNA specimen: label correct
  - Specimen reprep: Same cytology
Case 3

• **Clinical management**
  – Review of slides (diagnosis confirmed)
  – Referred to oncology
    • ? Radiation or chemotherapy
    • ? Additional surgery

• **Patient concerned about specimen mix-up**
Specimen Mix-up

- Legal issues
- Treatment issues
  - Patient
  - Other patients
Case 3

- Cytology specimen re-prep
- Block examined
  - Slide matches block
- Grossing schedule
- Clinical correlation
Case 3

Question:
Did the cancer in the FNA sample come from this patient?
Ancillary Tests

- Immunohistochemistry
  - ABO blood group antigens
- FISH for X and Y
Case 3

- Molecular identity testing
Case 3

• Compare DNA fingerprint
  – Patient
  – Specimens of interest
  – Potential other sources
Case 3

• Other important applications
  – Floater or carry-over artifact
Sample preparation

- Documentation
  - Slide digitization
  - Photographs
- Microdissection
Microdissection

- By hand
  - Microscope
  - Implement
- Laser capture
Microscope
Implement
Microdissection

Tumor Area 1

Tumor Area 2

Tumor Area 1

Tumor Area 2
Analysis of DNA

- Southern blots
  - Restriction fragment length polymorphisms (RFLP)
  - Need a lot of DNA from fresh tissue
Molecular Analysis

- Fresh tissue, blood, or cytology fluids
- Frozen tissue
- Fixed tissue
- Paraffin embedded tissue
  - Archival
Polymerase Chain Reaction

- Discovered in 1983
- Patented technique
- Patent sold to Roche in 1991 for $300 Million
- PCR licenses managed by Perkin-Elmer
  - 9% of collection
Polymerase Chain Reaction

- **Problems before PCR**
  - Abundance
  - Separation

- **PCR allowed**
  - Amplification
  - Specificity
Polymerase Chain Reaction

- Taq enzyme
- Primers
- Template DNA
- Nucleotide building blocks
Polymerase Chain Reaction

• Taq Enzyme
  – *Thermus Aquaticus*
  – DNA polymerase
  – Heat activated
Polymerase Chain Reaction

• Primers
  – Short DNA sequence (18-24 nucleotides)
  – Forward and Reverse
    • “Sense & Antisense”
PCR Primers

ATCGATTTCGAGACCCAG
GCATAGCTAAAGCTCTGGGTCAATTTTTAGAGTCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTA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PCR Primer Design
PCR Primer Design

• Computer program
  – Optimize primers
    • Avoid Hairpins
    • Avoid primer dimers
## Nucleotide Building Blocks

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</tr>
</tbody>
</table>
PCR Mechanics

- Cycling temperatures
  - Thermocycler machine
PCR Mechanics

- **Denaturation:** ~ 90º C
- **Annealing:** ~ 55º C
- **Extension:** ~ 72º C
PCR Amplification

PCR Product

Time
Case 3

- Theory for DNA fingerprinting
  - DNA Polymorphisms
DNA Fingerprinting

- Accepted forensic panel
- Polymorphic loci
- Loss mutations
ABI Identifier Kit*

- 16 polymorphic loci
  - Multiplex → 1 PCR
- Analysis with capillary electrophoresis
Identifiler Results

- 5 Different Dyes
- 16 Genetic Loci
Breast Lumpectomy

FNA specimen

Patient Blood
Breast Lumpectomy

FNA specimen

Patient Blood
## Case 3

<table>
<thead>
<tr>
<th>Target</th>
<th>D8s 1179</th>
<th>D21s 11</th>
<th>D7s 820</th>
<th>CSF 1PO</th>
<th>D3s 1358</th>
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<tr>
<td>Breast Biopsy</td>
<td>13, 14</td>
<td>30.2, 31.2</td>
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<td>15</td>
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<td>29, 32</td>
<td>11, 12</td>
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<td>30.2, 31.2</td>
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<td>30.2, 31.2</td>
<td>10, 11</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>
Case 3

• Diagnosis
  – Specimen mix-up
DNA Fingerprinting

• When we use this test
  – Any malignant floater
  – When diagnosis would be changed
  – Specimen mislabeling
  – Unexpected benign resection
Statistical Calculations

- **Unmatched**: None needed
- **Matched**
  - Depends on allele frequency
  - Example: \( \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = \frac{1}{1024} \)
Follow-up

• Risk Management involved
  – Blood test of all patient’s with FNA that day

• Laboratory
  – Benign FNA cell blocks from same day
Result--One Benign FNA
DNA Fingerprinting

• Other diagnostic applications
  – Transplantation analysis
    • Measure chimerism
    • Tumors in transplant patients
  – Maternal contamination in amniotic fluid
  – Paternity testing
  – Forensic applications
Case Four
Case Four

• 37 year old female
  – No prenatal care
  – HCG markedly elevated
  – Therapeutic abortion
Case Four

- **Pathology**
  - Hydropic villi
  - No fetal parts identified
Case

• **Differential diagnosis**
  – Hydropic abortion
  – Partial Hydatidiform Mole
  – Complete Hydatidiform Mole
Gestational Trophoblastic Disease

- **Hydropic abortion**
  - Sporadic genetic changes, or normal

- **Partial Mole**
  - 69 X,X,Y
  - Triploid

- **Complete Mole**
  - 46 X,X
  - Paternal chromosomal material
Analysis

- H&E and history
- Immunohistochemistry ($p57^{kip2}$)
- In situ hybridization
- Cytogenetics
- Molecular genotyping
Mole
Maternal
Hydropic Abortion

- Diploid
- 46 X,X or X,Y
- Grossly normal karyotype
- Normal fertilization
Inheritance Pattern
## Results

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
<th>Locus 4</th>
<th>Locus 5</th>
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<tbody>
<tr>
<td>Maternal</td>
<td>14, 17</td>
<td>6, 7</td>
<td>11</td>
<td>11, 12</td>
<td>17, 20</td>
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<tr>
<td>Villi</td>
<td>14, 17</td>
<td>6, 9</td>
<td>11</td>
<td>11, 12</td>
<td>17</td>
</tr>
<tr>
<td>Paternal</td>
<td>14, 17</td>
<td>6, 9</td>
<td>11, 12</td>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>
Partial Mole

- Triploid
- 69 X,X,Y or X,X,X
- One ovum fertilized by two sperm
Partial Mole
Complete Mole

- Diploid
- 46 X,X or X,Y
- Empty ovum fertilized by sperm
  - Haploid genome duplicates
  - Monospermatic (80%)
  - Dispermatic (20%)
- Paternal only genome
Monospermatic Pattern
## Results

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
<th>Locus 4</th>
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<tbody>
<tr>
<td>Maternal</td>
<td>10, 12</td>
<td>30, 31.2</td>
<td>10</td>
<td>12, 15</td>
</tr>
<tr>
<td>Villi</td>
<td>10</td>
<td>30</td>
<td>12</td>
<td>13</td>
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</table>
Dispermatic Pattern
## Results

<table>
<thead>
<tr>
<th>Specimen</th>
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<th>Locus 3</th>
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<th>Locus 5</th>
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<tbody>
<tr>
<td>Maternal</td>
<td>15, 16</td>
<td>6, 8</td>
<td>8, 12</td>
<td>11, 12</td>
<td>17, 18</td>
</tr>
<tr>
<td>Villi</td>
<td>15</td>
<td>7</td>
<td>10, 12</td>
<td>13</td>
<td>19, 20</td>
</tr>
</tbody>
</table>
Molecular Profile

- Advantages
  - Paternal vs. maternal alleles
  - Mono- vs. dispermatic moles
  - Prospective samples
  - Archived samples
  - Mosaic patterns
<table>
<thead>
<tr>
<th>Test</th>
<th>Spontaneous Abortion</th>
<th>Partial Mole</th>
<th>Complete Mole</th>
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<tbody>
<tr>
<td>Cytogenetics</td>
<td>Diploid</td>
<td>Triploid</td>
<td>Diploid</td>
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<tr>
<td>FISH</td>
<td>Diploid</td>
<td>Trisomy</td>
<td>Diploid</td>
</tr>
<tr>
<td>Immuno-stain</td>
<td>Expression</td>
<td>Expression</td>
<td>No Expression</td>
</tr>
<tr>
<td>Molecular Profiling</td>
<td>Diploid, biparental</td>
<td>Trisomy</td>
<td>Paternal only alleles</td>
</tr>
</tbody>
</table>
Maternal Contamination
Maternal Contamination
Case Five
Case 5

- Techniques illustrated
  - Microsatellite instability
Case 5

- 27 year old male
  - Emergency room
    - Colonic obstruction
    - Mass in abdomen
    - 25-lb weight loss
  - History
    - Father died of colon ca in 40’s
Case 5

- Colonoscopy
  - Large obstructing mass in right colon
  - Three other polyps

- Biopsies
  - Adenocarcinoma (mass)
  - Adenomas
Case 5

• Resection
  – 10 cm colonic adenocarcinoma
  – Duke’s Stage B
  – 3.5 cm tubular adenoma with high grade dysplasia

• Lymph nodes all negative (0/37)
Diagnostic Workup

- **Factors influencing workup**
  - Young age (<45)
  - Family history
  - Location and Histology

Genetic or Hereditary Origin?
Genetics of Colon Ca

• Tumor Suppressor Gene inactivation (85%)
  – APC gene (Vogelstein model)
  – Other genes: p53, p16, etc.

• Microsatellite instability (15%)
  – 30% Hereditary (Germline)
  – 70% Sporadic (Somatic)
Germline vs. Somatic

• Germline
  – In all cells
  – Started at the germ cell level
  – Can be inherited or spontaneous
  – Implications for the family members

• Somatic
  – Occurs in differentiated cells
  – No inheritance, no familial pattern
Microsatellite Instability

- DNA mis-match repair defects
Hereditary Mis-Match Repair Defect

- Hereditary nonpolyposis colorectal carcinoma syndrome
- Turcot syndrome
  - HNPCC
  - GBM
- Muir-Torre syndrome
  - HNPCC
  - Sebaceous skin tumors
  - Keratoacanthomas
HNPCC

- Incidence is 1 in 1,000
- Incidence in colon cancer is 1 in 100
  - 1-5% of all colon cancers
Who to test?

- Amsterdam criteria (I and II)
- Bethesda Guidelines
- Revised Bethesda Guidelines
Revised Bethesda

- **Under age 50**
- **Synchronous or metachronous tumors**
  - Colon CA or other HNPCC tumors at any age
- **MSI-High and under age 60**
- **Family History**
  - 1 or more 1\textsuperscript{st} degree relatives with Colon CA or other HNPCC tumor under age 50 or adenoma under 40
  - 2+ relatives with Colon CA or other HNPCC tumor any age
Pathology

- Right-sided
- Mucinous tumors
- Inflammatory reaction

MSI Testing?
Why test?

• To identify germline mutations
  – Early family screening
  – Second tumors in proband
• MSI High $\rightarrow$ better prognosis
• MSI High $\rightarrow$ responds to 5-FU
HNPCC

- Colon Cancer 80%
- Endometrial 50-60%
- Gastric 13%
- Ovarian 12%
Microsatellite Instability

- DNA mis-match repair mutations
- Epigenetic gene silencing
- Unknown causes
HNPCC

- Mutation in DNA mis-match repair gene
  - MSH2
  - MLH1
  - MSH6
  - PMS2
  - PMS1

Most Mutations
DNA replication

Polymerase

(5’ of template)

A — C — G

(3’)

Polymerase

A — T — T — A

(3’)

T — A — A — T

(5’ of copy)

G — C
Polymerase

DNA replication

(5’ of template)

Polymerase

(3’)

A

C

G

A

T

T

A

C

T

A

T

A

(3’)

(5’ of copy)

T

G

233
DNA replication

Polymerase

(5’ of template)

A - C - G - A - T - T - A

Polymerase

(3’)

T - A - A - T

(3’)

G - A

(5’ of copy)

DNA Mis-Match Repair

(5’ of template)

A  C  G  T

T  G  C  A

(3’)

T  A

(5’ of copy)

MSH 2

MLH 1

PMS 2

MSH 3/6
Unstable Areas

- Short tandem repeats
  - Particularly CA repeats

CA → CA → CA → CA → CA → CA
Unstable Areas

GT - GT - GT - GT - GT - GT
CA - CA - CA - CA - CA - CA

Polymerase

CA - CA
Mis-Match Repair

MSH 2

GT - GT - GT - GT - GT - GT

CA - CA - CA - CA - CA - CA

MLH 1

6 repeats

PMS 2

6 repeats

MSH 3/6
Microsatellite Instability

- 6 repeats
- 7 repeats
- 8 repeats
- 9 repeats
- 4 repeats
- 5 repeats
Microsatellite Instability

Normal Cell

Tumor

6, 8

6, 9

5, 10

8, 8

6, 5

6, 8

7, 8

5, 8

6, 6

4, 5

7, 9

5, 9

6, 6
Mis-Match Repair Defects

- 100-1000-fold increase in mutation rate
- Tumor progression is faster
MSI Testing

- PCR analysis of microsatellites
  - Number of repeats in normal
  - Number of repeats in tumor

- Novel sized PCR amplicons in tumor
Detection of MSI

- **Protein level**
  - Loss of expression of enzymes
    - MSH-2, MLH-1, MSH-6

- **Functional level**
  - Microsatellite analysis

- **Genomic level**
  - Gene mutations
Detection of MSI

- **Protein level**
  - ~75% Sn
  - 75-100% Sp

- **Microsatellite analysis**
  - ~100% Sn
  - ~40% Sp

- **Gene mutations**
MSI Testing Guidelines

- Panel of 5 microsatellite markers
  - BAT 25
  - BAT 26
  - D2s123
  - D5s346
  - D17s250
Mononucleotide Repeats

CTATGTATAAAAAAAAAAAAAAAAAGGCTCTAG
Dinucleotide Repeats

CTATGTATCACACACACACGGCTCTCTAG
Tetranucleotide Repeats

CTATGTATGAAGGAAGGAAGGAAGGAAGGGGCTCTAG
## Case 4

<table>
<thead>
<tr>
<th></th>
<th>BAT 25</th>
<th>BAT 26</th>
<th>D2s123</th>
<th>D5s346</th>
<th>D17s250</th>
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<tbody>
<tr>
<td>Polyp</td>
<td>MSI</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Tumor</td>
<td>MSI</td>
<td>MSI</td>
<td>MSI</td>
<td>MSI</td>
<td>Normal</td>
</tr>
</tbody>
</table>
Microsatellite Instability

D2s123
Interpretation of Results

• MSI High
  – 2 of 5 loci
  – >30% loci

• MSI Low
  – 1 of 5 loci
  – <30% loci

• Microsatellite Stable
  – 0 loci
Case 4

• **Tumor showed MSI at 4/5 loci tested**
  – MSI-High
• **Adenoma showed MSI at 1/5 loci**
Gene Mutation Analysis

- Sequencing of MLH-1 and MSH-2 genes
Genetic Implications

- MSI testing is not a genetic test
  - Somatic mutations
- Immunohistochemistry: Debated
  - Correlates highly with germline mutations
Protocol for MSI Testing

Pathologist Orders MSI & IHC

Report Result

Informed Consent

Germline Blood Test

Recommend Genetic Counseling

Report Results

Negative

Positive
Other Applications

- Young people with other HNPCC-related tumors
  - Gastric carcinoma
  - Endometrial carcinoma
- Other tumors with DNA mismatch repair
Case 4 Follow-up

- Germline mutation identified subsequently
Clinical Implications

- **Patient**
  - At risk for other tumors
- **Family**
  - At risk for hereditary transmission
Case Six
Case 6

- Techniques illustrated
  - Gene loss
  - Prognosis value of mutation analysis
Case 6

- 1993 – Brain tumor resected
  - Oligodendroglialoma
- 1999 – Recurrence
- 2001 – Recurrence
Gliomas—Issues

• Predict treatment response
  – Specific chromosomal losses
Measuring Chromosomal Loss

- Loss of heterozygosity
- FISH
- Comparative genomic hybridization
1p & 19q Markers

1p
- D1s1161
- D1s162
- D1s199
- D1s407

19q
- D19s400
- D19s559
- D19s918
- D19s206
Loss of Heterozygosity

- Brain biopsy – little normal
- Other normal surrogates
  - Buccal brush
  - Fingernail
  - Hair
Results

Buccal Brush | Fingernail | Hair | Licked Envelope
Results

- Buccal Brush
- Fingernail
- Hair
- Licked Envelope
## Results

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1s1161</td>
<td>1p35.1</td>
</tr>
<tr>
<td>D1s162</td>
<td>1p35.1</td>
</tr>
<tr>
<td>D1s199</td>
<td>1p36.12</td>
</tr>
<tr>
<td>D1s407</td>
<td>1p36.21</td>
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### Results

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Band</th>
<th>Status</th>
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<tbody>
<tr>
<td>D1s1172</td>
<td>1p22</td>
<td>NI</td>
</tr>
<tr>
<td>D1s226</td>
<td>1p22.1</td>
<td>NI</td>
</tr>
<tr>
<td>D1s1161</td>
<td>1p35.1</td>
<td>LOH</td>
</tr>
<tr>
<td>D1s162</td>
<td>1p35.1</td>
<td>LOH</td>
</tr>
<tr>
<td>D1s199</td>
<td>1p36.12</td>
<td>NI</td>
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<tr>
<td>D1s407</td>
<td>1p36.21</td>
<td>LOH</td>
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<tr>
<td>D1s1183</td>
<td>1q25</td>
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Results

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>D19s400</td>
<td>19q13.2</td>
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<td>D19s559</td>
<td>19q13.2</td>
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<tr>
<td>D19s918</td>
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<td>D19s112</td>
<td>19q13.32</td>
</tr>
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<td>D19s206</td>
<td>19q13.33</td>
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<td>Locus</td>
<td>chromosome arm</td>
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<tr>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>D19s561</td>
<td>19q11</td>
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<tr>
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<td>19q13.33</td>
</tr>
<tr>
<td>D19s401</td>
<td>19p11</td>
</tr>
</tbody>
</table>
Results: 1p

- Normal
- Allele 1: 1999
- Allele 2: 2001

Comparison for different years:
- 1999: Allele 1 > Allele 2
- 2001: Allele 1 < Allele 2
Problems With LOH

- Assumes equal amplification
  - Not always true: Allelic dropout
- Lack of normal to compare
- Mixture of normal and tumor cells
Allelic Dropout

Normal—PCR number one
Peaks are equal

Normal—PCR number two
Peak two is decreased
Allelic Dropout Conditions

- Very low DNA concentrations
- Allele 1 & Allele 2 very different in length
Unequal Amplification

Time

X

X

X

X
Allelic Dropout

Size of Microdissection Target (mm)

Allele Ratio

5.5 4.5 3.25 2 1.25 0.75
Interpreting LOH

• Lack of normal sample
  – Normalized normal
  – Primer and repeat length dependent
Primer Trials

delta = 4.64 + 3.23 * ratio
R-Square = 0.16
Example Gene

![Bar chart showing the mean allele ratio for different repeat length differences](chart.png)

- Mean Allele Ratio
- Repeat Length Difference

The chart displays the mean allele ratio for different repeat length differences. The x-axis represents the repeat length difference, and the y-axis represents the mean allele ratio.
Other Methods

- **FISH**
  - Probes for 1p and 19q
  - Good correlation with LOH (90-100%)

- **CGH**
  - Weaker correlation with LOH (50-80%)
FISH

• Paraffin sectioning artifact
  – Nuclear truncation
  – Use of whole nuclei
Genotyping

- Tumor progression in recurrence
<table>
<thead>
<tr>
<th>TSG</th>
<th>p16</th>
<th>p16</th>
<th>pten</th>
<th>pten</th>
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</thead>
<tbody>
<tr>
<td>1999</td>
<td>No</td>
<td>NI</td>
<td>No</td>
<td>NI</td>
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<tr>
<td>2001</td>
<td>LOH</td>
<td>NI</td>
<td>No</td>
<td>NI</td>
<td>No</td>
<td>LOH</td>
</tr>
</tbody>
</table>
Other Mutations

- Higher grade gliomas
  - P53 mutation
  - Losses in pten and p16
  - EGFR amplification
Therapeutic Implications

- 1p and 19q loss
  - Associated with oligodendroglial differentiation
  - Chemotherapy sensitivity
  - Prolonged survival

- Retention of 1p and 19q
  - Chemotherapy resistance

- Added mutations
  - Higher grade and worse prognosis
Molecular Anatomic Pathology

- Molecular Testing applied to AP
“Lasagna Diagnostics”
Assessing Value Added

- Advance understanding
- Diagnostic use
- Therapeutic use
- Prognostic use

Clinical Role
“Lasagna Diagnostics”

Integrated
Validated
Quality controlled
Case Summary

- **Neoplasia**
  - Double Primary
  - Thyroid tumor

- **Identity testing**
  - Specimen mix-up
  - Molar Pregnancy

- **Prognostics and therapeutics**
  - MSI
  - 1p and 19q loss
Methods Summary

- Loss of Heterozygosity
- DNA Fingerprinting
- Translocation analysis (RT-PCR)
- Microdissection
- Microsatellite instability
Thank You