Molecular Diagnosis for Colorectal Cancer Patients
Antonia R. Sepulveda MD, PhD, FCAP

October, 20, 2010
Welcome to the PHC Webinar Series

This talk on “The Molecular Diagnosis for Colorectal Cancer” is presented by Antonia Sepulveda, MD, PhD, FCAP.

Your host is Jill Kaufman, PhD. For comments or suggestions, please contact Jill Kaufman at jkaufma@cap.org

THE WEBINAR WILL BEGIN MOMENTARILY. ENJOY!
Antonia Sepulveda, MD, PhD, FCAP

- Director of Surgical Pathology, Hospital of the University of Pennsylvania

- Director of Surgical Pathology fellowship, Hospital of the University of Pennsylvania

- Medical Director, Molecular Test Development, Anatomic Pathology, Hospital of the University of Pennsylvania

- Member and Consultant, Morphology core, Center for Molecular Studies in Digestive and Liver Diseases, University of Pennsylvania School of Medicine

- Professor of Pathology and Laboratory Medicine at the Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine
Ongoing research in Dr. Sepulveda’s laboratory is focused on two main areas: The first is the study of the molecular mechanisms of H. pylori associated gastric cancer development. In addition, she has been a co-investigator in a number of studies involving animal models, providing pathology expertise in the evaluation of mouse tissues, with focus on diseases of the gastrointestinal tract.
Colorectal Cancer Burden and Outcomes

- Estimated new cases and deaths from colon and rectal cancer in the United States in 2010
- New cases: 102,900 (colon); 39,670 (rectal)
- Deaths: 51,370 (colon and rectal combined)
- Frequently CRC patients develop metastatic disease (stage IV)
  - Poor prognosis of metastatic CRC (<10% 5Y OS)
- These figures indicate a need for improved therapies and diagnostic tools

Therapeutic Modalities for Colorectal Cancer and the Role of Molecular Diagnosis

- Conventional therapies:
  5FU/Leucovorin; FOLFOX, (oxaliplatin); FOLFIR (Irinotecan)

- New therapies: Antibodies approved for metastatic colon cancer
  - Anti-EGFR: Molecular testing available to identify patients who benefit from therapy
    - Cetuximab (Erbitux)
      - FDA approved as second line therapy for CRC
    - Panitumumab (Vectibix)
      - FDA approved as third line therapy for CRC
  - Anti-VEGF: Bevacizumab (Avastin)- No specific molecular testing available
Molecular Diagnosis for Colorectal Cancer: Current Status

Currently available and widely used:

- Testing Tumor Tissues for selection of targeted therapies
  - Anti-EGFR antibody therapies
- Testing tumor tissues for DNA mismatch repair / microsatellite instability to help identify HNPCC/Lynch syndrome

Other tests available or in development:

- Testing tumor tissues for predictive markers for conventional chemotherapy
- Testing tumor tissues for prognostic markers
Colon Cancer Underlying Molecular Pathways Identify Subtypes of Colorectal Cancer

Sepulveda A. and Aisner D.
In: Molecular Pathology: The Molecular Basis of Human Disease, 2009.
Four Major Molecular Subtypes of Colorectal Cancer According to MSI and CIMP Status

MSI-H
CIMP-low/0
Gr. 2 (5%)

MSI-H
CIMP-H
Gr. 1 (10%)

MSI-L/MSS
CIMP-H
Gr. 3 (5-10%)

MSI-H
CIMP-high

MSI-L/MSS
CIMP-low/0
Gr. 4, 5, 6 (75-80%)

Shuji Ogino and Ajay Goel, JMD, 2008
Role and Expression Pattern of Epidermal Growth Factor Receptor in Colon Cancer

- EGFR (Her1, c-Erb-B1) is a transmembrane receptor
- EGFR is activated by several ligands (EGF, TGF-alpha)
- Upon ligand binding EGFR is activated and induces intracellular signaling cascades that lead to increased cell proliferation
- EGFR is expressed in normal colon epithelium and in 80-100% of colorectal cancers (by IHC)
EGFR EXPRESSION IN CRC: Immunohistochemistry
EGFR Signaling Pathway in Colorectal Cancer

Gazdar NEJM 2009
Anti-EGFR

EGF

EGFR

FAK

Src

Src

Grb2

SOS

Ras

Kras\textsuperscript{mut}

MEK

Erk/MAPK

PTEN

PI3K

Akt

mTOR

Cell Cycle progression

Gene Transcription

Proliferation

Cell Cycle progression

Current Opinion in Pharmacology
Mutations in EGFR Pathway Genes in Colorectal Cancer

Figure A and B show Venn diagrams illustrating the distribution of mutations in various genes involved in the EGFR pathway in colorectal cancer.

De Rook et al. Lancet Oncol 2010
Epidermal Growth Factor Receptor (EGFR) Targeted Therapy In Colorectal Cancer
Patients with metastatic CRC may benefit from anti-EGFR MoAbs Cetuximab and panitumumab therapies

- **Based on** Phase II & III clinical trials
- **Metastatic CRC** (Stage IV: any T, any N, M1)
- Using these MoABs as monotherapy or in combination with chemotherapy
- Tumors with KRAS mutation in codons 12 or 13 did **NOT** benefit from treatment with cetuximab or panitumumab
- Up to 40% response rate to anti-EGFR in wild type tumors
  - Remainder 60% wild type tumors will not respond: due to other altered mechanisms affecting signaling pathways
- Patients with metastatic CRC who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a CLIA-accredited laboratory

KRAS Mutation Status is Predictive of Response to anti-EGFR Therapy

Molecular Testing for EGFR Targeted Therapy in CRC: Kras Mutations

- Point mutations in the Kras gene occur in approximately 40% of colorectal adenomas and cancers.
- The mutated oncogenic p21 ras proteins resist GTP hydrolysis and have constitutively active signaling function.
- Most Kras activating mutations occur in codon 12 and less often in codon 13:
  - 70-80% in codon 12
  - 20-30% in codon 13
- Rare mutations in codon 61.
- Mutations in codon 146 have been reported.
Selecting Tissue Block for Molecular Testing

- Select block of FFPE tissue with highest % of viable tumor and largest tumor area
- Adequate DNA amount obtained by pooling macrodissected tissue from multiple levels
- Biopsy may be preferable to resection specimen if resection was done after neoadjuvant therapy

Resection specimen

Biopsy
Kras mutation assay types

- No FDA approved tests currently
- Laboratory developed tests:
  - Sanger Sequencing
  - Allele Specific PCR (DxS Therascreen)
  - Melt curve analysis
  - Pyrosequencing
- Choice of assay in a laboratory is defined by which assay the laboratory validated and routinely uses.
KRAS Mutation Detection: Pyrosequencing
Specimen Sample for Kras Mutation Test: Primary vs Metastasis

- Kras mutations occur early in colorectal carcinogenesis
- Most clinical trials tested the primary tumor site
- Published studies show good correlation between Kras mutation status in primary vs. metastatic colon cancer
  - concordance 94-100%.
Improvement in Response Prediction by Assessing Mutation Status of Multiple EGFR-pathway Genes

De Rook et al. Lancet Oncol 2010
Response to Cetuximab Based Therapy and Status of Multiple EGFR-pathway Genes

- De Rook et al. study supports the negative effect of KRAS mutations on outcome after cetuximab therapy
- BRAF, NRAS, and PIK3CA exon 20 mutations are significantly associated with a low response rate
- Objective response rates could be improved by additional genotyping of BRAF, NRAS, and PIK3CA exon 20 mutations in a KRAS wild-type population

De Rook et al. Lancet Oncol 2010
Testing DNA Mismatch Repair in Colorectal Cancer-Clinical Applications

- **Widely used clinically:**
  - Workup for Lynch syndrome (LS/HNPCC)
- **Questionable clinical Application:**
  - Prognostic marker—Better survival of MSI-H tumors
  - Predict response to 5-FU based therapy: MSI-H tumors show lack of benefit
Molecular Testing in the Workup for Lynch Syndrome (LS/HNPCC)

- Sine qua non for diagnosis of Lynch syndrome is identification of a germline mutation in a DNA mismatch repair gene: MLH1, MSH2, MSH6, or PMS2 (peripheral blood test)

Testing Tumor Tissues in the Workup for Lynch Syndrome

• Pathogenic mutations of MMR genes result in DNA mismatch repair deficiency & underlie microsatellite instability (MSI) type mutation

• Molecular testing of tumor tissues useful in the workup of patients for LS:
  o IHC for DNA mismatch repair proteins in tumor cells: useful to determine which gene may be deficient
  o MSI testing of DNA from tumor tissue: surrogate marker of DNA mismatch repair deficiency

• MLH1 methylation and BRAF mutation tests: contributory in workup of MSI-positive tumors with loss of MLH1
Selecting Patients for Molecular Workup for Lynch Syndrome

- Bethesda Guidelines
  - Umar, A. et al. JNCI 96: 261-268, 2004

- Generalized Screening of CRC
  - Hampel H. J Natl Compr Canc Netw, 2010
Revised Bethesda Guidelines for LS/HNPCC and MSI Testing

1-CRC diagnosed in a patient less than 50 years of age.
2-CRC with MSI-H histology diagnosed in a patient less than 60 years of age.
3-Synchronous, metachronous CRC, or other LS/HNPCC-associated tumors.
4-Individual with CRC and at least one first degree relative with CRC/HNPCC tumor less than 50 years of age.
5-Patient with CRC and two or more first- or second-degree relatives with CRC/HNPCC-related tumors.
DNA MMR Deficient/MSI Cancers: LS/Hereditary and Sporadic Colorectal Cancers

CRC - MSS

CRC MSI-High: Sporadic 15%

MSI-High: LS/HNPCC 2-4%
Gross and Morphologic Features of MSI-H CRC

Gologan & Sepulveda et al.
Arch Pathol Lab Med 2005; 129: 1390-1397
<table>
<thead>
<tr>
<th>MMR genes affected in LS/HNPCC Tumors</th>
<th>Molecular Mechanism</th>
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<tbody>
<tr>
<td>MSH2</td>
<td>Germline mutations</td>
</tr>
<tr>
<td>MLH1</td>
<td>Approximately 40%</td>
</tr>
<tr>
<td>MSH6</td>
<td>Approximately 10%</td>
</tr>
<tr>
<td>PMS2</td>
<td>Approximately 5%</td>
</tr>
<tr>
<td>MLH3</td>
<td>disputed</td>
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<tr>
<td>MLH1 and MSH2</td>
<td>Germline Epimutation of promoter region (rare cases)</td>
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<tr>
<th>MMR genes affected in Sporadic MSI-CRC</th>
<th>Somatic Epigenetic alteration</th>
</tr>
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<tbody>
<tr>
<td>MLH1</td>
<td>Promoter hypermethylation /gene silencing</td>
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Selection of Tumor Tissues in the Workup for Lynch syndrome LS/HNPCC

• Colorectal cancer
• Endometrial and Ovarian cancer
• Indications for testing colonic adenomas
• Resection specimens vs. biopsies
What is MSI?

- MSI is a mutation that occurs in small (100-200 base pair) DNA segments of repetitive nucleotides, called microsatellite regions or short tandem repeats.

- Sequences of repetitive elements tested in MSI: adenine (A)n mononucleotide repeats or cytosine-adenine (CA)n dinucleotide repeats.

- During DNA replication these repetitive sequences may decrease or increase in length because of strand slippage: MSI type mutation.

A.R. Sepulveda, Medscape Pathology; Posted: 03/19/2008;
The Importance of Microsatellite Instability in Colonic Neoplasms (review)
Microsatellite Instability

122 base pairs

AGCCCGG A AAA A AAA AA(N) CTAAACCC

116 base pairs

AGCCCGG A AAA AA(N) CTAAACCC
TESTING MSI IN TUMORS
PROTOCOL

- Unstained sections from formalin fixed paraffin embedded tissue (5-7 micron); overlap with H&E stained section
- From same patient select one or two tissue blocks containing
  - Viable Tumor
  - Noneoplastic tissue
- Scrape selected areas for DNA extraction at least 1 cm²
  - Tumor Area (T); Nonneoplastic tissue (N)
TESTING MSI IN TUMORS
PROTOCOL

- PCR amplification with MSI panel
- DNA fragment characterization by electrophoresis: most common capillary electrophoresis
MSI TEST: NCI PANEL OF MICROSATELLITE MARKERS

- NCI 5 microsatellite marker panel
  - Mononucleotide repeat:
    - BAT 25 and BAT 26
  - Dinucleotide repeat:
    - D2S123, D5S346 and D17S250

Umar, A. et al. JNCI 96: 261-268, 2004
 MSI SUB-TYPES With NCI Panel

• High level MSI (MSI-H)
  o MSI in ≥ 30% of markers tested (at least 2 markers out of five)

• Low level MSI (MSI-L)
  o MSI in <30% of markers tested (only 1 MSI-positive marker)

• Microsatellite stable (MSS)
  o No markers show MSI
MSI TEST: Other panels of markers

- MSI Analysis System (Promega Corp.)
  - 5 mononucleotide markers
  - 2 pentanucleotide repeat markers
- Complete concordance with the NCI-panel of 5 markers for MSI-H and MSS tumors.
- MSI-L cases by the NCI panel were MSS when tested by the Promega MSI Analysis System.

Murphy KM et al & Eshleman JR. J Mol Diagn. 2006;8:305-311.
Performance of markers in NCI panel

Gologan & Sepulveda et al.
Arch Pathol Lab Med 2005; 129: 1390-1397
MSI-Mononucleotide Repeats

Note: 2 separate alleles in BAT 26

Occurs in up to 10% of A-Americans
MSI-Dinucleotide Repeats
DNA Mismatch Repair (MMR) Proteins

- **MutS**: MSH2, MSH6, MSH3
- **MutL**: MLH1, PMS2, MLH3

MMR proteins function as heterodimers. The heterodimers are required for protein stability.

<table>
<thead>
<tr>
<th>MutS</th>
<th>MutL</th>
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<tbody>
<tr>
<td>MSH2-MSH6</td>
<td>MLH1-PMS2</td>
</tr>
<tr>
<td>MSH2-MSH3</td>
<td>MLH1-MLH3</td>
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# Immunohistochemical Patterns in MSI-H CRC

<table>
<thead>
<tr>
<th></th>
<th>IHC MLH1</th>
<th>IHC PMS2</th>
<th>IHC MSH2</th>
<th>IHC MSH6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MLH1 Mutation</strong></td>
<td><strong>Loss</strong></td>
<td><strong>Loss</strong></td>
<td>Preserved</td>
<td>Preserved</td>
</tr>
<tr>
<td><strong>MSH2 Mutation</strong></td>
<td>Preserved</td>
<td>Preserved</td>
<td><strong>Loss</strong></td>
<td><strong>Loss</strong></td>
</tr>
<tr>
<td><strong>MSH6 Mutation</strong></td>
<td>Preserved</td>
<td>Preserved</td>
<td>Preserved</td>
<td><strong>Loss</strong></td>
</tr>
<tr>
<td><strong>PMS2 Mutation</strong></td>
<td>Preserved</td>
<td><strong>Loss</strong></td>
<td>Preserved</td>
<td>Preserved</td>
</tr>
</tbody>
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A.R. Sepulveda, Medscape Pathology; Posted: 03/19/2008; The Importance of Microsatellite Instability in Colonic Neoplasms (review)  
MSI test and IHC in Tumor Tissues

MSI-H & Loss DNA repair protein in tumor (MSH6)

MSS with Loss of DNA repair proteins in tumor (MSH6)

MSI-H & Preserved DNA repair proteins in tumor

MSI-H & Loss DNA repair protein in tumor

MSI-High correlates well with loss of expression of main DNA repair proteins (MLH1 or MSH2) by Immunohistochemistry.
Test (sensitivity/specificity) to identify Lynch syndrome

- MSI testing among those with *MLH1* or *MSH2* mutations: sensitivity 80-91%; specificity is 90%.
- MSI testing among those with *MSH6* (or *PMS2*) mutations: sensitivity 55–77%; specificity 90%.
- IHC testing, regardless of MMR gene involved: sensitivity 83%; specificity of 89%.

*EGAPP, Genet Med. 2009; 11(1): 35–41*
Example of MSI and IHC: MSI-H CRC with MSH2-Loss

IHC identification of primary deficient DNA repair gene

- 45 year old man
- Transverse colon obstructing mass
- Right hemicolecotomy: 10 cm exophytic mass (T3N0MX)
- IHC for MLH1, MSH2 and MSH6 performed
IHC identification of primary deficient DNA repair gene

MLH1

MSH2

MSH6
MSI and DNA Mismatch repair IHC in Colorectal Cancers: Biopsy vs. Resection

• Most commonly use resection specimen but biopsy is OK (may be the only available tissue)
• Using biopsy allows for selection of surgical procedure (partial vs. subtotal colectomy)
• MSI test with NCI panel requires control DNA from non-neoplastic tissue of same patient-if run without this control results may be inconclusive.
  o Alternatives include:
    - Obtaining constitutional DNA from other source (eg buccal swab)
    - Run another panel of markers (eg. MSI Analysis System Promega)
Testing MSI in Adenomas

• The combination of MSI and IHC testing in colorectal adenomas is a sensitive screening approach for the detection of Lynch syndrome (tested in LS patient group)

• Significant association between MSI and IHC protein loss and high-grade dysplasia
  o Pino et al. Journal of Molecular Diagnostics, Vol. 11, No. 3, 2009

• Testing MSI/DNA repair IHC in adenomas in the general population has not been supported by published literature
Additional Molecular Tests for Differential Diagnosis Between LS/HNPCC and Sporadic MSI CRC

• Most of the mutations in BRAF (activating mutations): thymine to adenine transversion at nucleotide position 1796
  o leading to the substitution of valine for glutamate (V600E)

• BRAF V600E:
  o Sporadic MSI-H CRC (40-74%); MSS CRC (4-12%)
  o Not detected in any LS/HNPCC tumors

DNA Sequence Analysis: BRAF Mutation

WT

Mut V600E
BRAF Test (sensitivity/specificity) to identify Lynch syndrome

- BRAF mutation testing is usually restricted to CRC cases with absent staining for MLH1
  - Lynch syndrome: Virtually 100% negative BRAF mutation
  - Sporadic CRC: 40-80% positive for BRAF mutation

Boland and Shike, Gastroenterology. 138, 2010
Quantification of MLH1 Methylation: Distinction of LS/HNPCC and Sporadic MSI-CRC

- LS/HNPCC patients showed no or low level of MLH1 promoter methylation in CRC
  - Sporadic MSI-H CRC with loss of MLH1 expression and BRAF V600E mutation:
    - All positive MLH1 CpG methylation (all >18% of reference)
  - Sporadic MSS CRC
    - No MLH1 promoter methylation
  - A cutoff value of 18% methylation was determined in this study to define MLH1 hypermethylation specific for sporadic MSI-H cases.

Quantification of MLH1 Methylation: Distinction of LS/HNPCC and Sporadic MSI-CRC

BRAF and MLH1 Methylation Tests in CRC

- In CRC’s with MSI and absent MLH1 expression, analysis of BRAF mutation and/or methylation of the MLH1 promoter is recommended.

Boland and Shike, Gastroenterology. 138, 2010
Meeting summary from the Jerusalem workshop on LS/HNPCC
Algorithm for Testing CRC for workup of Lynch Syndrome/HNPCC

Tumor Tissue:
Immunohistochemistry for MMR proteins
Or Microsatellite Instability Test (MSI)

MSI test: MSI-H
IHC: Loss of Expression of DNA MMR Protein(s)

Germline Testing of MMR Gene(s)
Identified by lost expression by IHC

Germline Mutation in MMR gene(s) Identified:
LS/HNPCC

If Loss of MLH1 by IHC:
BRAF Mutation Test
MLH1 Methylation Test

MLH1 Methylation Positive
And/or
BRAF Mutation Positive

Unlikely LS/HNPCC

Possible Undetected Mutation or Epimutation in MLH1

Negative
MLH1 Methylation Test
BRAF Mutation Test

Offer Genetic Counseling by Cancer Genetics Specialist to Family Relatives
Summary

**Testing CRC tumor tissues for patient selection for targeted anti-EGFR antibody therapies is widely used**
- Kras mutational analysis is recommended
- Mutational testing of other EGFR pathway genes may be helpful (more studies needed)

**Testing tumor tissues for DNA mismatch repair / microsatellite instability to help identify HNPCC/Lynch syndrome is widely used**
- Recent studies indicate that it may be cost effective to test all patients with newly diagnosed CRC

**Ongoing translational research and clinical trials are testing a number of predictive and prognostic markers in CRC**
- Not ready for prime time
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- **Molecular Diagnosis for Lung Cancer Patients** – Tuesday, November 9th, 10-11 am CT
  - Philip T. Cagle, MD, FCAP

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  - Introduction to the Medical Home
  - Personalized Medicine: Framing the Issues for Pathology
  - Clinical Requests for Molecular Tests