THE FUTURE OF PAP SMEAR SCREENING IN THE ERA OF MOLECULAR DIAGNOSTICS AND VACCINES

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ASIP COMPANION MEETING USACP 2009

TAKE HOME BULLET POINTS:

1. MORPHOLOGY BASED SCREENING HAS BEEN THE MODEL FOR CANCER SCREENING
2. THE MATHEMATICS OF SCREENING PREDICTS PROBLEMS WITH CONTINUING TO SCREEN ONLY WITH THE PAP
3. PROPHYLACTIC HPV VACCINES WILL COMPOUND AND ACCELERATE THESE ISSUES
4. SCREENING WITH MOLECULAR METHODS IS THE MOST LIKELY SOLUTION TO THESE PROBLEMS

INTRODUCTION

The conventional Papanicolaou (Pap) smear is the world's most successful cancer screening test. Improving knowledge of the interaction between human papillomaviruses (HPV) and the development of cervical neoplasia continues to impact cervical cancer screening practice. Yet, the concept of combining molecular testing with morphology for cervical screening is so revolutionary that the medical community continues to debate choices regarding cervical cancer screening and practice. In the United States, the adoption of HPV as an adjunctive primary screening test has been slow. The reasons for this lag in implementation are complex. They undoubtedly reflect the interplay among long-established clinical practice patterns, lack of education regarding the data supporting the recommendations for joint testing, and a lack of understanding of the potential medicolegal impact of not performing what may well be a superior mode of testing.

DIAGNOSTIC REPRODUCIBILTY: HPV FOR QUALITY CONTROL

Clearly diagnostic reproducibility is critical to the practice of pathology as well as to the clinical management of pathologically diagnosed cervical cancer and its precursors. Previous studies on the reproducibility of cervical neoplasia diagnosis are best characterized as limited in size and for the most part, statistically inadequate. The National Cancer Institute (NCI)-sponsored Atypical Squamous Cells of Undetermined Significance-Low-Grade Squamous Intraepithelial Lesion (ASCUS-LSIL) Triage Study (ALTS) was designed in part to compare reproducibility of both cytological and histological diagnoses.

During enrollment in the ALTS trial the pathologists interpreted 4,948 ThinPreps and 2,237 colposcopic biopsies. The comparisons between the clinical center and QC groups were performed in a completely masked manner. The conclusion of these analyses was that, regardless of specimen type, there was only moderate interobserver reproducibility between the pathologist at the clinical center and the QC pathology group. There was significant asymmetry...
in each class of comparison suggesting that there was a systematic pattern of disagreement between the clinical center and QC pathologists, with QC pathologists tending to give less severe interpretations in all three types of specimens.

Given this relatively low degree of reproducibility that exists even between expert pathologists, clinicians need to be aware of this source of variability as well as the compounding variability due to variations in colposcopic assessment and biopsy placement. Because of this, objective independent methods of assessing diagnostic accuracy such as HPV testing should assume a more prominent role in the management of patients with mild squamous abnormality on Papanicolaou smear.

**HPV TESTING AS AN ADJUNCT FOR TRIAGE: ALREADY A STANDARD**

ALTS firmly established adjunctive testing for high-risk HPV as the standard of care for the triage of patients with mildly abnormal cervical cytology. As noted above, high-risk HPV testing is directly correlated with the degree of cytologic interpretive certainty. What this means is that high-risk HPV testing can be used to control the quality of cytologic interpretations for an individual, group or laboratory. More importantly, the ALTS trial clearly demonstrated that HPV testing is the most effective method for the triage of patients who have an equivocal cytologic interpretation. The burden of ASCUS diagnosis on clinical practice is substantial. The critical practice question for physicians treating these patients is how to find the fraction of these patients who have significant high-grade precursor lesions. Complementary studies have clearly shown that the majority of high-grade lesions found in clinical practice actually come from the ASCUS group. Indeed, 60% of colposcopically derived biopsies read as high-grade cervical intraepithelial neoplasia (CIN) come from patients who have Pap smears read as either ASCUS or LSIL, yet ASCUS is an inherently irreproducible cytologic diagnosis. ASCUS is perhaps better viewed as a statistical risk group in which the clinical challenge is to find the high-grade CIN when the majority of these patients really harbor no evidence of clinical or colposcopic high-grade CIN. In a series of papers that have come to be known as the ALTS "quartet," the essential finding of the ALTS trial was that HPV triage was 92% sensitive for prevalent CIN3 in the ASC-US group while referring only 53% of patients for colposcopy. There was no combination of cytologic follow-up at any interpretive threshold that equaled this level of sensitivity while referring the same or fewer patients to colposcopy. Hence, HPV testing seemed to be the optimal triage method for detecting the critical high-grade disease in this clinically large population of patients. Of additional interest to the practicing clinician was the fact that HPV testing also improved the sensitivity of colposcopy. Specifically, knowledge of HPV status significantly improved the colposcopic sensitivity of biopsy. Before the ALTS trial, colposcopy was widely thought to be the gold standard for the detection of CIN. ALTS clearly demonstrated colposcopy to be less sensitive than was previously perceived. Finally, while HPV testing is an excellent triage method for equivocal cytology, in patients with cytologically diagnosed LSIL, high-risk HPV testing has little utility. This is because at least 84% of patients with LSIL on cytology are already high-risk HPV positive. Hence, HPV testing will not effectively triage these patients in whom it was established that there is a 25% to 30% likelihood of detecting a high-grade cancer precursor over 2 years.

Given the success of HPV testing for triage of patients with abnormal cytologic findings, the question naturally arises as to whether HPV testing should become the standard of care for primary screening. This is particularly relevant in patients older than the age of 30 in whom the prevalence of HPV is falling while the relative prevalence of significant histologic abnormality is increasing.
HPV TESTING FOR PRIMARY SCREENING: THE TIME HAS COME!

If one looks at the causes for cervical cancer screening failure in the United States, it is clear that more than 50% of the patients in whom cervical cancer develops have never been screened, and another 10% to 20% of those patients have not been screened in at least 5 years. However, at least 30% of the patients in whom cervical cancer develops have had a "false negative" Pap smear and approximately half of those false-negative results are due to sampling error while the remaining 50% actually are screening or interpretive errors. While society recognizes the success of the "imperfect" Pap smear in reducing the incidence of cervical cancer, the primacy of the Pap smear has been threatened because of the burden on the pathology community inflicted by the problem of false-negative results. Any single cervical cancer screening event may only be 50% sensitive for prevalent disease. Thus, it is only through the repetitive application of independent screening events, at relatively short intervals, that the Pap smear "system" is really efficacious. While it is possible for women who routinely have perfect annual screening attendance to have very low rates of cervical cancer, even with perfect attendance, the system is imperfect and false-negative results will continue to occur. So how do we minimize false-negative results while at the same time maximizing the efficiency and decreasing the inconvenience of the cervical cancer screening process? The practical possibilities include improving the quality of the sample, improving the detection of abnormal cells in the sample, improving the accuracy of morphologic interpretation, and taking morphology out of the equation. Thin-layer cytology has been widely implemented and in many reports increases in sensitivity and specificity have been achieved although this is controversial. One might further decrease the fatigue and consequent false-negative rate of screening cytology samples by the implementation of automation systems. This, too, has had some marginal impact on the rate of false-negative cytology. But both of these methods have not had the dramatic desired impact on false-negative rates needed to decrease the frequency of adverse medicolegal events. Assuming most of the optimization of cytology has already been completed, it is only logical to ask whether an independent test for the etiology of the disease that does not require the subjectivity of the entire cytologic process can improve the performance of primary screening.

The primary data supporting the utility of HPV testing in conjunction with cytology for screening consist of several studies that evaluated more than 60,000 women in a variety of clinical settings. All studies demonstrated that HPV testing was more sensitive than cervical cytology regardless of the clinical setting. At such high levels of sensitivity, many have been concerned that too many patients would be referred to colposcopy, and those referred would fail to have identifiable lesions. Surprisingly, HPV testing achieved specificities that were comparable to or only slightly less than those with cytology alone. This undoubtedly reflects the problem of interpretive variability inherent in morphologic assessment. HPV testing has been shown to be more sensitive than cytology, is more reproducible than cytology and, because of the high sensitivity of combined testing, the consequent high negative predictive value in a low prevalence population provides for less frequent screening, which ultimately may increase the compliance of patients with screening recommendations.

So why has HPV testing for primary screening made such a small impact on clinical practice? Clearly, clinicians are concerned about the need for counseling patients, especially regarding the significance of HPV positivity in the setting of a negative cytology. Perhaps a bigger concern is the change in practice surrounding interval extension. However, these concerns have to be balanced by societal and epidemiologic perspectives. The best strategy for preventing cervical cancer is to use the most accurate test at the longest possible interval.
MEDICOLEGAL CONCERNS:

The standard of care is established in professional communities based on consensus. This consensus can be brought about through the interaction of experts, medical literature and individual or local practice. Unfortunately, best-practice standards often conflict with other interests. The best test may not be the most affordable test. The system that leads to maximal liability protection may be in conflict with the needs of both payers and patients in terms of cost effectiveness vs. patient protection. Is the conventional dry-slide Pap smear still acceptable for cervical screening? Certainly, the "more-than-50%" rule in terms of standard of care would seem to suggest that conventional Pap smears are not the "standard of care" in many communities. Of course, the logical extension of such reasoning is to ask whether and when a laboratory will stop processing conventional Pap smears as "acceptable" specimens. In many academic settings where conventional Pap smears account for less than 5% of routine specimens, the ability to adequately train practitioners on the interpretation of conventional preparations may become questionable.

Likewise, there seems to be a large gap between the published data regarding HPV testing efficacy and the medical community's understanding of the complexities of the underlying biology and science. Is the need for HPV testing a patient, clinician, or laboratory judgment? Who determines the adequacy of a testing specimen? If a blood test is delivered in the wrong type of collection tube or in an inadequate volume, does the laboratory not have the right to reject the specimen? Up until the time of publication of the data supporting the FDA's approval of HPV testing as an adjunct for primary screening, it was clear that there seemed to be several ways to screen for cervical cancer. All methods, that is, conventional Pap smears, liquid-based cytology, and so on, were "accepted." However, some methods seem to have a significant false-negative rate. Clearly, from a legal perspective, false-negative results may cause a bad patient outcome. Bad patient outcomes ultimately result in bad financial outcomes for practitioners. The practitioners that seem most at risk are pathologists, because pathologists are responsible for accepting, processing, and rendering reports on specimens submitted to laboratories for cervical cancer screening.

Hence, from a legal perspective, it would seem that a testing paradigm that maximizes sensitivity while minimizing false-negative results would result in optimal patient care and minimize malpractice risk for pathology laboratories. Liquid-based cytology combined with HPV testing might be suggested (from the legal perspective) as the only modality acceptable for cervical cancer screening. In such a system, false-negative results would be virtually eliminated. Hence, screened women might be almost guaranteed to avoid invasive cervical cancer. Pathologists might get sued less frequently. The theoretical risk exposure for not following practice guidelines would be transferred to laboratories who continue to accept specimens that are less optimal or to gynecologists and other practitioners who fail to follow contemporary screening guidelines.

THE POSSIBLE NEAR FUTURE: HPV FIRST THEN (OR NO) CYTOLOGY

In the short term, combining HPV testing with cytology screening costs more money. Any system that costs more money has to overcome the inertia against increased expenditure that is inherent in our current payment system. The ultimate question, therefore, is whose interest is controlling: the interest of payers or the interest of patients, and, by extension, the physicians who treat those patients? If the data supporting the introduction of HPV testing as an adjunct to cervical cancer screening are valid, then it would seem that ultimately the best medicine for
patients should ultimately lead to better and more rational medical practice. Indeed, just-published data imply that using HPV testing to screen for cervical cancer could have a dramatic impact not only in the United States, but also on a worldwide basis.

Even more controversial but logical is the concept that HPV testing as the more sensitive test should be the primary screening test. Cytology would be a potential triage tool for HPV rather than the other way around. While potentially threatening to some such a move is not only a logical way to save money, but recent data suggest that it can be essentially as effective as doing both tests on all patients.

HPV VACCINES

Obviously human papillomaviruses are the etiologic agent for a significant spectrum of pathology. The ideal solution to this plague upon society would be the elimination of these viruses through prophylactic vaccination. The era of prophylactic vaccines is upon us. A large-scale phase III trials of bi-valent and quadrivalent VLP vaccine against HPV 16/18 or HPVs 6/11/16 and 18 are ongoing, but the FDA data for the per protocol analysis demonstrated 100% efficacy for all cervical and vulvar pathologies related to the vaccine types Ultimately multivalent vaccines seem likely. The implications of societal HPV vaccination on screening systems, lesional incidence, cost-benefit analyses, etc. are complex, stimulating and are sure to fuel vigorous debate for years to come.

SELECTED READINGS

Besides the individual references below a comprehensive useful compilation of contemporary thinking can be found in:


Cox JT, Schiffman M, Solomon D. Prospective follow-up suggests similar risk of subsequent cervical intraepithelial neoplasia grade 2 or 3 among women with cervical intraepithelial neoplasia grade 1 or negative colposcopy and directed biopsy. Am J Obstet Gynecol. 2003;188:1406-1412.
“Recent advances in the molecular pathology of lung cancer: role of EGFR and KRAS mutation testing in treatment selection”

Marc Ladanyi, M.D.
Memorial Sloan-Kettering Cancer Center
New York, NY

Web resources

The Sanger Institute (U.K.) maintains the COSMIC database (Catalogue Of Somatic Mutations In Cancer) that includes a listing of all reported EGFR mutations, as well as mutations in KRAS and other kinases:
http://www.sanger.ac.uk/genetics/CGP/cosmic/

The City of Hope Clinical Molecular Diagnostic Laboratory (Duarte, CA) maintains an EGFR mutation database:
http://mdl.cityofhope.org/egfr_db/index.html

Recent reviews and articles


“Recent advances in the molecular pathology of lung cancer: role of EGFR and KRAS mutation testing in treatment selection”

Marc Ladanyi, M.D.
Chief, Molecular Diagnostics Service
Memorial Sloan-Kettering Cancer Center
New York, NY, USA

Predictive molecular testing for targeted therapy of lung cancers

1. Testing for EGFR and KRAS mutations in Lung Cancer
2. Beyond EGFR and KRAS...

EGFR

- EGFR is a receptor tyrosine kinase of the ErbB family
- 4 closely related receptors
  - ErbB1 – EGFR, HER1
  - ErbB2 – HER2/neu
  - ErbB3 – HER3
  - ErbB4 – HER4

Small molecule EGFR tyrosine kinase inhibitors (TKIs)

- Oral drugs with relatively low toxicity (rash, diarrhea)
- Pharmacokinetics – once-daily dosing
- Expression of EGFR by IHC in large proportion (50-60%) of lung CA provided initial rationale for trials
Dramatic response to gefitinib

February 6, 2002
February 11, 2002

Histologic features of responders to EGFR inhibitors

- Responders:
  - typically well to moderately differentiated adenocarcinoma
  - peripheral; TTF1+
  - loosely associated with BAC or adenocarcinoma with BAC component
- Non-responders:
  - more often poorly differentiated; more often TTF1-negative
  - pure squamous carcinomas (in contrast to adenosquamous CA)
  - mucinous BAC (assoc with KRAS mutations)
- Histologic overlap between the 2 populations
  - Among lung adenocarcinomas, negative predictive value of morphologic features insufficient to exclude EGFR mutation testing

Histologic features of responders to EGFR inhibitors

- Responders:
  - typically well to moderately differentiated adenocarcinoma
  - BAC components, papillary component
  - peripheral; TTF1+
  - often with (non-mucinous) BAC component
- Non-responders:
  - more often poorly differentiated; more often TTF1-negative
  - pure squamous carcinomas (in contrast to adenosquamous CA)
  - mucinous BAC (assoc with KRAS mutations)
- Histologic overlap between the 2 populations

Discovery of activating mutations in EGFR kinase domain in lung cancers (2004)

EGFR Mutations Associated with Sensitivity to EGFR-TKIs

EGFR Mutations and EGFR TKI Responses

- Data cumulated by Sequist et al., JCO March 2007
- EGFR mutations:
  - Among responders = ~80%
  - Among non-responders = 7%
    - Reasons: ? Not all EGFR mutations confer sensitivity, ? Other mutations (e.g. PTEN loss, PIK3CA mut, etc.)
- Responses:
  - Among EGFR-mutant cases = ~80%
  - Among EGFR-mutation negative cases = 10%
    - Reasons: ? Undetected EGFR mutations due to limited extent or poor sensitivity of direct sequencing
    - Direct sequencing is likely to miss mutations when the tumor cells make up less than 25% of the sample
Association between EGFR mutation and EGFR Amplification

- Difficult to tease out because of their frequent co-occurrence.
- Reported ranges vary depending on technique, criteria and interobserver variability.
- Among EGFR mutated cases, ~50% show increased EGFR copy number.
- ~75% of cases with increased EGFR copy number show mutations.

Mutation status may be more rational for treatment selection

- The response rates to EGFR TKIs are high in the EGFR mutated case regardless of the copy number.
- The EGFR amplified cases that lack mutations have very low response rates in the order on 1-8%.
- When both alterations are present, the mutated allele is preferentially amplified suggesting that it is the mutation what drives the selection for copy number gains.

Phase II trial of erlotinib in 101 pts with BAC or adenocarcinoma, BAC subtype

<table>
<thead>
<tr>
<th>EGFR Status</th>
<th>n</th>
<th>Response (%)</th>
<th>Median PFS (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut+/CISH ≥ 4</td>
<td>10</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>Mut+/CISH &lt; 4</td>
<td>7</td>
<td>71</td>
<td>13</td>
</tr>
<tr>
<td>Mut-/CISH ≥ 4</td>
<td>14</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mut-/CISH &lt; 4</td>
<td>45</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>


- Mutant EGFR is biologically linked to ligand independent activation and increased downstream signaling.
- EGFR mutations more closely linked to known risk factors and patient profile (female, Asian, non-smoker) than is EGFR amplification.
Pts with EGFR Mutations Survive Longer on Gefitinib – Prospective studies


Iressa Pan Asian Study (iPASS)


Overall EGFR mutation positive rate = 59.7% (261 / 437) !!


M+, mutation positive;  M-, mutation negative


Dramatic responses to EGFR inhibitor gefitinib, but usually not durable


Durable responses (>3 yrs) in some, but most patients progress after 6-12 months.


PFS, progression-free survival; ITT, intent-to-treat


iPASS - Study Details


iPASS - Comparison of PFS by mutation status within treatment arms


Gefitinib EGFR M+ (n=132)  Gefitinib EGFR M- (n=199)
Carboplatin / paclitaxel EGFR M+ (n=132)  Carboplatin / paclitaxel EGFR M- (n=199)


Gefitinib, HR=0.19, 95% CI 0.13, 0.26, p<0.0001
No. events M+ = 97 (73.5%)  No. events M- = 88 (96.7%)


Carboplatin / paclitaxel, HR=0.78, 95% CI 0.57, 1.06, p=0.1103
No. events M+ = 111 (86.0%)  No. events M- = 70 (82.4%)


M+, mutation positive;  M-, mutation negative


1 month


1 month


Tony Mok
Resistance to EGFR-TKIs

- Primary resistance
  - Tumors that are refractory to EGFR TKIs from the outset
  - Genetic predictors at diagnosis
    - KRAS mutations (common); PTEN loss?
    - EGFR mutations not sensitive to EGFR TKIs (rare, ~2%)
      - ex 20 insertion
    - BRAF mutations (rare, ~3%)
      - Most, if not all, initial responders
      - Genetic finding:
        - second EGFR mutation: T790M (most)
        - MET amplification (some)
- Secondary resistance
  - Tumors that are initially sensitive to EGFR TKIs but eventually progress while on therapy
  - Genetic finding:
    - second EGFR mutation: T790M and ex20 insertions (and mutant HER2)

Mutations in the EGFR Pathway in lung adenocarcinoma

KRAS Mutations: A Negative Predictor for Response to EGFR TKIs in lung cancer

BUT, while KRAS mutations are found in about 15-30% of American lung adenocarcinomas, they are found in only <10% of Asian samples


KRAS patient profile compared to EGFR

- Smoking history more common
- No association with BAC histology (except mucinous BAC)
- Less common in Asians than non-Asians

Does it matter? EGFR and KRAS mutations: predictors of survival in resected lung adenocarcinoma

All Lung Cancers resected at MSKCC

Clinical EGFR/KRAS testing at MSKCC: current flowchart

Adenocarcinoma

No testing

EGFR mutation testing

KRAS mutation testing

Adenocarcinoma pts seen by Thoracic Oncologists

Special request only

EGFR initiated late 2004

KRAS initiated late 2006

15-20/week; 750/year

Outside pts: in 2007, 700 advanced stage pts through Thoracic Oncology/1/3 come with 15 unstained slides or blocks

Operated pts: reflex testing in 1 year period:

500 lung resections

297 adenocas

295 (98%) studied for EGFR/KRAS

58 (20%) EGFR

85 (28%) KRAS

Adenocarcinoma pts seen by Thoracic Oncologists

EGFR ex. 19 deletion assay

Lab of Diagnostic Molecular Pathology, MSKCC

- approx. 45% of EGFR mutations
- 9, 12, 15, 18, or 24 bp deletions (all preserve reading frame)
- most often 15 bp deletion eliminating amino acids EL REA

EGFR ex. 21 L858R mutation assay

Lab of Diagnostic Molecular Pathology, MSKCC

- approx. 40% of EGFR mutations
- substitution of leucine by arginine at codon 858 (L858R)

KRAS mutation assay (PCR-sequencing)

Lab of Diagnostic Molecular Pathology, MSKCC

F

GTT > GCT

G12A

R
Limitations of mutation detection by direct sequencing

- Sequencing will not detect proportions of tumor cells below the sensitivity level (25%).
- Microdissection routinely used to increase tumor content (eliminate non-neoplastic areas)
- Blocks or unstained sections for DNA extraction should be from the most cellular areas with >50% tumor cells.
- Select sections without excessive inflammatory response.

Use of *EGFR* and *KRAS* mutation status to select EGFR-TKI therapy

<table>
<thead>
<tr>
<th>Scenario</th>
<th><em>EGFR</em> Mutation</th>
<th><em>KRAS</em> Mutation</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>Gefitinib or Erlotinib</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Trial of drug?</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>Alternative agents</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>Extremely rare, if real</td>
</tr>
</tbody>
</table>

"The Lung Adenocarcinoma Oncogenome"

Mutually exclusive mutations: *EGFR, KRAS, BRAF, ERBB2*

**Mutations in the EGFR Pathway in lung adenocarcinoma**

**BRAF MUTATIONS in Lung CA**

- *BRAF* mutations identified in 2-3 % of lung adenocarcinoma
- Most common mutation is V600E, Exon 15
- Predicts resistance to EGFR inhibition
- Predicts benefit from MEK selective inhibition

(Pratillas et al., Cancer Res 68:9375–83, 2008)
Predictive molecular testing for targeted therapy of lung cancers

1. Testing for EGFR and KRAS mutations in Lung Cancer

2. Beyond EGFR and KRAS...

Predictive Testing for Targeted Therapies: impact on clinical labs

- New emphasis on mutation detection
- Methods need to work on DNA extracted from archival pathology paraffin blocks
- Methods need to detect mutations even when tumor cells are <10% of tumor biopsy sample
- Increasing need for higher-throughput molecular diagnostic technologies

A New Way to Look at Lung Cancer

>5 genes x multiple different mutations/gene = lots of assays → 1 mutation / assay no longer sustainable for clinical labs

Next generation molecular diagnostics: screening for multiple mutations more efficiently

- What’s needed: high throughput / high sensitivity
  - Multiplexing (multiple mutations tested / assay)
  - Automated liquid handling (reduce labor costs)
  - Need to detect mutations even when tumor cells are only 5% (or less) of tumor biopsy sample
  - Semi-automated mutation calling would be nice...

- New technology in place at MSKCC since 2006:
  - Sequenom: instrument for highly multiplexed mass spectrometry-based nucleic acid assays
Sequenom system
Mass spectrometry-based nucleic acid assays

Sequenom assays for lung kinase mutations

Clinical Genes in Sequenom Panel example: KRAS & EGFR

Research Genes in Sequenom Panel example: BRAF & PIK3CA

Clinical / Research tumor mutation profiling

Predictive Testing for Targeted Therapies: impact on clinical trials
Predictive molecular testing for targeted therapy of lung cancers

1. Testing for EGFR and KRAS mutations in Lung Cancer

2. Beyond EGFR and KRAS...

Acknowledgements

- Some slides graciously provided by:
  - William Pao, MD PhD
  - Maria Arcila, MD
The Role of miRNAs in Cancer Diagnosis and Prognosis: No Small Matter

Marina N. Nikiforova, M.D.
Division of Molecular Anatomic Pathology
University of Pittsburgh Medical Center
Pittsburgh, PA

Outline
- miRNA nomenclature, structure and function
- miRNA detection techniques
- miRNA dysregulation in human cancers
- Potential diagnostic, prognostic and therapeutic use

MicroRNAs (miRNAs)
- miRNAs are a new class of small (19-24 nt), non-protein-coding, endogenous RNA molecules
- Function as negative regulators of coding gene expression
- Regulate mRNA expression and prevent protein production through the RNAi pathway

miRNA History
- First discovered in 1993 by Victor Ambros and Gary Ruvkun (lin-4 of C. elegans)
- Next discovered miRNA was let-7 in 2000
- Currently, 695 miRNAs registered in online database (mirBase, Sanger Institute), more than 1000 predicted

miRNA Biology
- miRNA genes estimated to account up to 2-5% of human genes and regulates ~30% of coding genes
- Located within introns of protein-coding or non-coding genes, in exons of non-coding genes, or in 3'UTRs
- Chromosomal clustering (54 miRNA genes on chromosome 19)
miRNA and Cancer

- miRNA affect genes responsible for cell proliferation and apoptosis
- More than half of miRNA genes located in cancer-associated regions, including regions of LOH and amplification, and at chromosome fragile sites
- miRNAs may function as oncogenes or tumor suppressors

Techniques for miRNA detection

- Northern Blot analysis
- Microarray profiling
- RT-PCR

Comparison of miRNA and siRNA

<table>
<thead>
<tr>
<th></th>
<th>miRNA</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>18-24 nt</td>
<td>18-24 nt</td>
</tr>
<tr>
<td>Origin</td>
<td>Endogenous (miRNA genes)</td>
<td>Endogenous or exogenous ds RNA (no siRNA genes)</td>
</tr>
<tr>
<td>Conservation</td>
<td>Phylogenetically conserved</td>
<td>No conservation</td>
</tr>
<tr>
<td>Target</td>
<td>Regulate thousands of endogenous genes</td>
<td>Few targets, usually exogenous (i.e. viral)</td>
</tr>
<tr>
<td>Effect</td>
<td>miRNA cleavage or translational repression</td>
<td>miRNA cleavage</td>
</tr>
</tbody>
</table>

Suppressor miRNAs and Oncogenic miRNAs

Northern Blot

- Gold standard, detects miRNA precursors and mature miRNAs
- 5-10 ug total RNA required, low-throughput, low sensitivity for low-abundance species
miRNA Microarrays

- Slide (microchip) based technique
- Bead-based flow cytometric technique
  - High-throughput techniques, 2.5-5 ug total RNA required
  - Good sensitivity and specificity

Real-time RT-PCR

- Microarray or individual assays
- Performed in two steps:
  1) RT using stem-loop target-specific primers
  2) Real-time PCR
- 1-10 ng per reaction
- High sensitivity and specificity
- Ideal for FFPE tissue

miRNAs in Frozen vs. FFPE tissue

- FFPE tissue can be used for miRNA profiling
- Expression is similar, but proportionately lower as compared to frozen tissues

miRNAs in Normal Tissue

- At least 1/3 of miRNAs are tissue specific (miR-122 expressed in liver, miR-375 in pancreas, miR-142 and miR-223 in hematopoietic system, miR-1 in muscle)
- miRNA expression profiles allows to determine tissue origin

miRNAs in Cancer Tissue

- miRNA expression is generally downregulated in tumors as compared to normal tissues
- miRNA profiles in tumor tissues are different from normal tissues, but remain tissue specific
- miRNA profiling can be used for cancer diagnosis
miRNA expression profiles allow successful classification of poorly differentiated tumors

Jun Lu et al. 2005 Nature 435: 834-838

**miRNA as Diagnostic and Prognostic Tool**

- Establishing diagnosis of cancer
- Differentiate tumor from benign lesions
- Determine origin of poorly differentiated tumors
- miRNA expression can predict clinical behavior of certain cancers and disease outcome
- Some miRNAs associated with tumor progression (higher grade, tumor recurrence, metastasis) and serve as markers of poor prognosis

**Pancreatic Cancer**

- 65 ductal ACs and 42 chronic pancreatitis specimens analyzed for 326 miRNAs
- Most upregulated miR-21, miR-221, miR-222, miR-181a, miR-181b, miR-181d, and miR-155
- 23 miRNAs correctly differentiate ductal ACs from normal pancreas and chronic pancreatitis with 93% accuracy

**Thyroid Cancer**

- 60 thyroid tumors and 62 thyroid FNA samples studied for expression of 158 miRNAs
- 32% upregulated and 38% downregulated (>2 folds)
- miRNAs profiles correlate with cell origin and histologic type of tumors
- Most overexpressed miRNAs: miR-221, miR-222, miR-146b, miR-187, miR-155, miR-197, miR-224

**Clinical Utility of miRNAs**

- Diagnosis
- Prognosis
- Therapeutic application

**Pancreatic Cancer**

Kaplan-Meier Overall Survival Curve

**Thyroid Cancer**

Panel of 7 miRNAs for Diagnosis of Thyroid Cancer

- miR-187
- miR-221
- miR-222
- miR-146b
- miR-155
- miR-197
- miR-224

**Molecular Pathways in Thyroid Papillary Cancer**

- Tall cell and classic PC
- Independent prognostic marker of tumor recurrence and mortality
- Follicular variant of PC
- Classic histology PC

**Thyroid Cancer: FNA Samples**

- 6 PCs with local tumor recurrence or distant metastases (aggressive PCs) and 6 PCs with no complications on similar length follow-up (non-aggressive PCs)
- Matched by sex, age, and mutational status
- Studied for expression of 328 miRNAs

**Thyroid Cancer: Prediction of Aggressiveness**

- Upregulated miRNAs
Thyroid Cancer: Prediction of Aggressiveness

Downregulated miRNAs

- let-7 miRNA was differentially expressed in NSCLC
- Patients with low let-7 show significantly shorter survival
- let-7 family negatively regulates RAS

Lung Cancer

- 43 miRNAs distinguish lung cancer from non-cancerous lung tissue
- Upregulation of miR-155 and downregulation of let-7a-2 correlate with poor survival
- miR-155 is an independent prognostic factor

miRNAs as Therapeutic Targets

- Oncogenic miRNAs can be targeted for downregulation using anti-sense oligonucleotides
- Tumor suppressive miRNAs may be directly upregulated for anti-cancer effect
- Several studies have shown potential therapeutic effect of miRs and anti-miRs in vitro and in vivo
- Delivery of let-7 inhibits growth of human lung cancer cell lines and reduce tumor formation in mouse lung (Esquela-Kerscher et al. 2008 Cell Cycle)
- Challenge in development of effective drug delivery
Summary

- miRNAs function as negative regulators of coding gene expression
- miRNAs are tissue specific
- miRNA expression is dysregulated in human cancers; can be exploited for tumor diagnosis (surgical and FNA) and prognostication
- miRNAs may serve as a promising target for development of novel anti-cancer therapies
By integrating morphology and molecular pathology, pathologists can play a role in three crucial issues that may improve the colorectal cancer management: the assessment of an individual's colorectal cancer risk, indication and/or evaluation of neoadjuvant therapies and the assessment of individual's molecular targeted therapies.

a) INDIVIDUAL’S COLORECTAL CANCER RISK:

One out of each 17 persons will develop a colorectal cancer (average risk) but 25% of cases of colorectal cancer (CRC) occur in individuals who have above average or high risk for colorectal cancer. Individuals with one or more first-degree relatives with colorectal cancer have a relative risk for colorectal cancer of 2.25 and 4.25, respectively. This risk is even higher for familial and hereditary colon cancer syndromes. Patients with inherited germline mutations of hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (accounts for approximately 5% of cases of colorectal cancer) have an 80% lifetime cancer risk and patients with Familial Adenomatous Polyposis (FAP) a 100% cancer risk. To identify the 6-8% of all colorectal cancers occurring in a familial or hereditary onset is crucial prevention and early detection of other tumours associated with these syndromes and for genetic testing of the families to detect mutation carriers that should undergo rigorous cancer screening, starting at 20 years of age.

HNPCC is the most frequent hereditary syndrome associated with colorectal cancer. Affected individuals carry a germline mutation of mismatch repair (MMR) genes (MLH1, MSH2, MSH6 or PMS2) and a somatic genetic second hit originates loss of expression of the MMR gene and microsatellite instability (MSI). Molecular studies to identify HNPCC can be recommended with a meticulous family history and the early onset of tumours, but still some cases
may be missed. Some authors have proposed screening of all colorectal cancer tumors for microsatellite instability. There is a good correlation between the loss of MMR protein expression by immunohistochemistry and microsatellite instability identification by PCR. A cost effective strategy for HNPCC screening is the analysis of MMR gene expression by IHC and/or microsatellite instability and only positive cases undergo genetic testing for mutations in mismatch repair genes MLH1 and MSH2. Pathologists can integrate clinical data, IHC results and MSI data to identify those patients. MMR genes function as heterodimers (MLH1-PMS2 and MLH2-MSH6). Loss of protein expression usually affects two MMR genes: the protein of the mutated MMR gene and its heterodimer protein. Positive staining for inflammatory and stromal cells can be used as an internal IHC control.

Gross examination of colorectal cancer specimen can detect more than 100 adenomatous polyps characteristic of FAP, although attenuated forms of FAP may have fewer polyps and a later onset (mean age 35 years-old for classic FAP and 50 years-old for attenuated forms).

Individuals with inflammatory bowel disease, adenomas with dysplasia and traditional or sessile serrated adenomas also have a higher CRC risk. During the past decade, major advances have occurred in our understanding of the molecular basis of CRC and its precursor lesions. There is increasing evidence for an alternative pathway of sporadic colorectal tumourigenesis other than the adenoma-carcinoma sequence. A serrated polyp pathway that is associated with DNA microsatellite instability (MSI), due to methylation and loss of expression of the mismatch repair gene MLH1 with activating mutations in BRAF has been highlighted. However, for many pathologists the lack of consensus criteria in morphologic parameters makes difficult to distinguish hyperplastic polyps and the spectrum of serrated mucosal lesions.

b) THE TNM STAGING SYSTEM:

The TNM was established as a prognostic system but now determines individual treatment. A same patient can receive or not neoadjuvant therapy or
may enter into a clinical trial depending on which version of the TNM system is being used.

- Number of identified lymph nodes. In most institutions, CRC surgical resection specimens with less than 12 identified lymph nodes will receive chemotherapy even if no lymph node metastases are found. Recent large scale studies show that increased fixation time (over 36 hours with formalin) can increase between 10-15% the number of specimens with more than 12 lymph nodes. A fat clearance technique for the detection of lymph nodes in colorectal cancer is been used at some institutions. The fixatives used in this technique can interfere with DNA extraction and when used, we recommend procuring frozen or formalin fixed tumour and normal tissues for molecular studies.

- Tumoural deposits in the fat: The TNM version 6 distinguishes independent deposits with a round contour that should be considered as lymph node metastasis from irregularly shaped deposits that should be considered an extension of the T category (V1 as microscopic vascular invasion and V2 as gross vascular invasion), although some discrepancies have been reported about the prognostic significance of these deposits in some series.

- Micrometastasis: groups measuring less than 0.2 mm are considered as non relevant.

c) CIRCUMFERENTIAL RESECTION MARGIN (CRM) AND TOTAL MESORECTAL EXTRIPATION (TME) EVALUATION IN RECTAL ADENOCARCINOMA:

The surgical margin of resection is crucial for determining the prognosis and survival in rectum. A 2008 review showed in a series of 17,568 patients that the assessment of CRM margin has a greater prognostic influence that the depth of infiltration of the primary tumor, regardless of neoadjuvant therapy. Pathological evaluation of the CRM was described by Quirke et al. and more recently by Nagtegaal et al. It is defined as the margin of soft tissue or perineal adventicia
closer to the point of maximum penetration of the tumour or lymph node metastasis. It is considered to be positive if it is <1 mm. Despite these data, the report of the CRM is not a routine practice in many laboratories. The evaluation of the circumferential margin is even more important after preoperative treatment when the radiological study shows a positive margin.

Related to circumferential margin is the evaluation of the level of quality of surgery over the usual total mesorectal excision (TME), which is valued from grade 1 to 3 (3= complete resection). There are controversies over its usefulness in some recent clinical trials.

d) RESPONSE TO PRE-SURGICAL CHEMO-RADIOThERAPY:

Several clinical studies have highlighted the importance of a grading system to assess tumour regression in response to preoperative chemotherapy or radiotherapy treatment in rectal cancer. Four degrees of tumour regression (TRG) can be determined based on the proportion of viable tumour versus fibrosis: TRG 4 there is no viable tumour, TRG 3: regression greater than 50%, TRG 2 less than 50% and TRG 1: tumour unchanged.

e) INDIVIDUAL'S MOLECULAR TARGETED THERAPIES:

K-ras mutations and advanced CRC treatment with EGFR inhibitors: Most colorectal cancers (up to 80%) have increased expression of Epidermal Growth Factor receptors (EGFR) and use this molecular pathway as a mechanism for tumour growth. Blocking EGFR with monoclonal antibodies is a useful cancer therapy only when K-ras oncoprotein is “wild type”. Kras is located downstream in the EGFR growth stimulation molecular pathway (ras / MAPK pathway) and when kras is mutated it will continue to activate this pathway even if EGFR are blocked. Kras mutations are present in 40-45% of all colorectal cancers (47% in our first 200 advanced CRC cases analyzed for EGFR inhibitors treatment). Clinical trials results presented at the ASCO in June 2008 and the World Congress of Gastro-intestinal cancer in Barcelona 2008 demonstrated in large multicenter clinical trials that metastatic CRC with wildtype Kras (non-mutated) can benefit from therapy with anti-EGFR monoclonal antibodies such as Amgen.
Vectibix (panitumumab) and Erbitux from Imclone / Bristol-Myers Squibb (cetuximab), as a second line treatment. Results of clinical trials using EGFR inhibitors as a first line treatment alone or associated with chemotherapy or angiogenesis inhibitors will be presented in ASCO 2009.

Currently, a network to use EGFR inhibitors as a second line treatment in all advanced CRC has been set up in Spain as a multi-level, European pioneer for cancer treatment. Endoscopy material, tumour paraffin blocks or cytological FNAB are submitted to a few reference hospitals as the Hospital San Carlos to assess the kras mutation status. The pathologist can play an important role in the selection of tumour material for DNA extraction, valuing the percentage of neoplastic cells or the need for microdissection. Each molecular technique that can be used to analyze kras status has a different tissue requirement and a different mutation sensibility.

References.

- Christos Karapetis1, Shirin Khambata-Ford2, et al: KRAS Mutation status is a predictive biomarker for cetuximab benefit in the treatment of advanced colorectal cancer - Results from NCIC CTG CO.17: A phase III trial of cetuximab versus best supportive care.
- Australasian Gastrointestinal Trials Group, National Health and Medical Research Council Clinical Trials Centre, University of Sydney, Sydney, NSW, Australia; 2Bristol-Myers-Squibb Company, Princeton NJ & Wallingford CT, USA; 3National Cancer Institute of Canada Clinical Trials Group, Queen’s University, Kingston, ON, Canada. Abstract ASCO 2008.