Prediction is difficult, especially about the future

Niels Bohr, 1885-1962

Traditional trial-and-error method of care is no longer acceptable

What does the consumer want?

- High quality
- Reasonable cost
- Delivery as fast as possible
- Minimal inconvenience
- Access to care with the latest technology
- Reduced risk
- Confidence and trust

What does the patient’s treating physician want?

Help!

- Fast and accurate results
- Understandable and useful information
- Direction on therapy
- Low costs--may not be as important
Market demand and emerging technologies are accelerating the shift to “Precision” medicine

- Provision of care for diseases which can be precisely diagnosed and subsequently treated with predictably effective rules-based therapies
  - Precision technologies driving the disruption of existing healthcare business models
  - **Precise diagnosis must precede predictably effective therapy**
- Requires technology progress on two fronts
  - Understanding the cause of disease
  - Ability to detect those casual factors

**Source:** Christensen/Hwang

---

Today, Cancer is experiencing a similar shift toward precision medicine

- 2 types: leukemia & lymphoma
- 3 types of leukemia (acute, chronic, preleukemia) and 2 types of lymphoma (indolent, aggressive)
- 38 types of leukemia; 51 types of lymphoma

**Precision medicine is at the core of personalization**

**Source:** Mara Aspinall, Genzyme

---

Industry recognizes the opportunity and are willing to work with anyone.

**Are diagnostics the new wonder drug on Wall Street?**

---

Diagnostics and knowledge integration are the critical links to the success of personalized medicine

**Prognosis & Treatment**

- Gene Expression
- Pharmacogenomics
- Biomarkers
- Traditional Pathology
- **The value of traditional pathology has not diminished. It simply is no longer be sufficient.**

**Predisposition, Signs, Symptoms**

---

The cost of diagnosing and treating infectious diseases has declined 5% per year since 1940

**Source:** Christensen/Hwang
Pathology will assume a critical role in health care delivery.

**Pathologists**
- Have a unique knowledge of disease processes
- Are knowledge integrators
- Can get access to all the diagnostic data necessary
- Are responsible for the testing that is driving therapy

Vision of Pathologists

Pathologists are physicians who take an active role in patient care, utilizing all available tools to integrate and interpret diagnostic information to provide an accurate diagnosis of disease. Pathologists work closely with other members of the medical team to assess the patient condition and prognosis in order to determine optimum therapy alternatives.

Re-assess your tool kit—all diagnostic tools are available to you
- Acknowledge market forces driving changes in practice of pathology
- Be life long learners
- Expand beyond the tissue on the slide
- Investigate new Dx tools that will bring value to your patients

Expand your sphere of influence; take the central role in the treatment team
- Market your services for consults
  - Establish and advertise an open door policy
  - Invite your clinician peers to meet with you to review slides, case histories and interpretations
- Take a more active role in influencing prognosis and treatment; Communicate!
  - Identify and sponsor educational opportunities that demonstrate integration of pathology and the rest of the treatment plan
  - Collaborate with others providing diagnostic data
  - Bring new Dx solutions to the table; educate your clinician peers on the incremental value to patient care
- Go see patients
  - Actively participate in patient grand rounds
  - Review charts and talk to the attending physicians
EGFR Assays in Lung Cancer
Sanja Dacic, MD, PhD
University of Pittsburgh Medical Center

OUTLINE

• Development of targeted therapies in lung cancer
• EGFR testing in lung carcinoma
  – DNA sequencing, FISH/CISH, immunohistochemistry
• Role of surgical pathologists in targeted therapies

LUNG CANCER STATISTICS

Five-Year Relative Survival Rates(%) by Sex and Cancer Site, 1998-2000
All Races

© 2008 College of American Pathologists. Materials are used with the permission of the faculty.
Why EGFR?

• 170-kDa membrane bound protein encoded by 28 exons on chromosome 7p21
• TK (tyrosine-kinase) family
• HER subfamily
  – EGFR: HER1 (c-ErbB1)
  – HER2-neu (c-ErbB2)
  – HER3
  – HER4

EXTRACELLULAR DOMAIN
TRANSMEMBRANE DOMAIN
INTRACELLULAR DOMAIN

KEY DISCOVERIES ABOUT EGFR

• Involved in cell signaling
• Oncogene
• Overexpressed in various cancers
• Activity could be inhibited by monoclonal antibodies
Monoclonal Ab (Erbitux)
Small molecule inhibitors (Iressa, Tarceva)

Figure 3. Example of the Response to Gefitinib in a Patient with Refractory Non-Small-Cell Lung Cancer. A computed tomographic scan of the chest in Patient A shows a large mass in the right lung before treatment with gefitinib was begun (Panel A) and marked improvement six weeks after gefitinib was initiated (Panel B).

How to identify patients who might benefit from targeted therapies?

© 2008 College of American Pathologists. Materials are used with the permission of the faculty.
EGFR mutations strongly correlate with clinical response to TKIs, but are not the only factor.

What histologic type of adenocarcinoma should be tested?
- Probably all adenocarcinomas of the lung, regardless of histologic subtype, should be tested.

What assay to choose?

- Fresh, frozen or paraffin embedded tumor tissue
- Tumor microdissection
- DNA extraction
- DNA sequencing
Mutations may not be detected by direct sequencing if tumor cells make up <25% of the sample.

Samples obtained after multiple courses of chemotherapy.

**OTHER MUTATION DETECTION TECHNIQUES**

- SSCP (Marchetti et al. JCO 2005;23:857)

**ALTERNATIVE APPROACHES TO PREDICT SENSITIVITY TO TKIs**

- Immunohistochemistry
- EGFR gene amplification

**FISH/CISH or MUTATION**

- EGFR mutations frequently associated with increased EGFR gene copy numbers

---

Do other mutations predict tumor response to TKIs?

- KRAS mutations are strong negative predictor of tumor response to EGFR TKIs
  - Strongly associated with smoking
  - Less common in Asians than non-Asians

How to predict a secondary resistance to TKIs?

- EGFR mutated tumors show high response rate regardless of amplification status
- EGFR amplified tumors negative for mutations show low response rate
- EGFR amplified tumors negative for mutations show survival benefit for EGFR TKIs
Second mutation in exon 20 (T790M)

Amplification of MET gene (7q31)

What is the role of surgical pathologist in EGFR testing?

• diagnosis of non-small cell carcinoma, NOS in surgical pathology reports is not acceptable any more
• to ensure that only adenocarcinoma/adenosquamous carcinomas are tested
• to select the most cellular blocks or unstained sections for mutational analysis (>50% tumor cells)

TAKE HOME MESSAGE

• Clinical parameters of response/sensitivity to TKI therapies in lung carcinoma are relatively well defined
• EGFR mutations are not the only genetic factor of the tumor response to TKI therapies
• EGFR and KRAS mutational analysis by DNA sequencing and EGFR gene amplification by FISH, are currently adopted assays in many academic centers

FUTURE DIRECTIONS -1

• One assay may not be sufficient to determine therapeutic options for patients with lung adenocarcinoma
FUTURE DIRECTIONS – 2

- Laboratory tests may not be necessary to determine therapeutic options for patients with lung adenocarcinoma.

Questions?
EGFR signaling network plays a central role in the development of many cancers including non-small cell lung carcinoma (NSCLC) and is recognized as a target for the development of anti-NSCLC treatments.

Small molecules, including gefitinib and erlotinib, were developed to inhibit EGFR signaling by blocking the intracellular tyrosine kinase (TK) domain.

Initial clinical trials reported objective responses to EGFR TKIs in 10-27% of NSCLC patients after failure of chemotherapy.

Clinical features associated with increased response rate to EGFR TKIs include female gender, never smokers, Asian ethnicity and adenocarcinoma histology.

Somatic mutations in the exons 18-21 of the TK domain of EGFR correlate with a high likelihood of response to EGFR TKIs and observed clinical characteristics of responders.

The most common are in-frame deletions in exon 19 (45%) and point mutation (CTG to CGG) in exon 21 at nucleotide 2573 which results in substitution of leucine by arginine at codon 858 (L858R) (41%).

EGFR mutations are not the only genetic factor of the tumor response to TKI therapies.

KRAS mutations are predictor of failure of EGFR TKI therapy. They occur in adenocarcinomas of smokers and are adverse prognostic factor.

EGFR mutation analysis is currently accepted as the most accurate test for prediction of response to EGFR TKI. Combining EGFR mutation analysis with EGFR FISH or CISH may provide additional useful information.

Almost all patients who respond to EGFR TKIs develop resistance.

Acquired resistance to EGFR TKIs is associated with a second mutation, T790M in exon 20 of the TK domain, and MET amplification.

The pathologist role in EGFR testing is to ensure that testing is performed only in adenocarcinomas or adenosquamous carcinoma, unless specifically requested by oncologist, and to select a paraffin block for mutation analysis with >50% of tumor cells.
Lung cancer is the leading cause of cancer-related death in the world. Non-small cell lung carcinoma (NSCLC) accounts for 80-85% of all lung cancers with only 15% 5-year survival. Most of patients present with advanced-stage disease, and cytotoxic chemotherapy is the only therapeutic option, which provides an improvement in survival of only 2 to 4 months. Therefore, there is a need for development of new therapeutic strategies in lung cancer.

The EGFR signaling network plays a central role in the development of many cancers including NSCLC and was recognized as a target for the development of anti-NSCLC treatments. The epidermal growth factor receptor (EGFR, HER-1/ErbB1) is a member of the ErbB family of tyrosine kinase receptors (TK), which includes HER-1/ErbB1, HER-2/neu/ErbB2, HER-3/ErbB3 and HER-4/ErbB4. It is composed of extracellular (ligand binding), transmembrane and intracellular (tyrosine-kinase) domain. Upon ligand binding and receptor homo-or hetero-dimerization and activation, activated EGFR signals downstream to the PI3K/AKT and RAS/RAF/MAPK pathways. These intracellular signaling pathways regulate key processes such as apoptosis and proliferation. EGFR is expressed in a large proportion of epithelial tumors and its role in lung cancer has been known for decades.

Small molecules, including gefitinib and erlotinib, were developed to inhibit EGFR signaling by blocking the intracellular TK domain. Initial clinical trials reported objective responses to EGFR TKIs in 10-27% of NSCLC patients after failure of chemotherapy. A randomized phase III trial comparing erlotinib to placebo (NCIC BR-21) showed survival benefit of erlotinib, while similar study comparing gefitinib to placebo failed to show survival advantage. In both of the studies, at least 30% of patients showed no clinical benefit. Several clinical features were found to be associated with increased response rate to EGFR TKIs including female gender, never smokers, Asian ethnicity and adenocarcinoma histology, particularly one with features of bronchioloalveolar carcinoma. (1-17)

In 2004, three academic groups almost simultaneously reported the discovery of somatic mutations in the exons 18-21 of the TK domain of EGFR that correlated with a high likelihood of response to EGFR TKIs and observed clinical characteristics of responders. (18-20)The most common are in-frame deletions in exon 19 (45%), followed by a point mutation (CTG to CGG) in exon 21 at nucleotide 2573 which results in substitution of leucine by arginine at codon 858 (L858R) (41%). Other less common mutations, which are associated with sensitivity to EGFR TKIs, include G719 mutations in exon 18 and the L861 mutations in exon 21. The incidence of EGFR mutations in Asians is 25-50% and 10% in Western patients. The strongest predictor of mutation status is absent or low smoking history. It seems that EGFR mutations are limited to lung cancer, and initially no mutations have been identified in other types of cancer. However, recently rare missense mutations in exons 19 and 21 were detected in colorectal carcinoma. (21,22) The same deletions in exon 19 as seen in lung cancer were also detected in squamous cell carcinoma of the head and neck. (23)

It was clear from the experience with targeted therapy for the breast cancer that a new standardized assay procedures for assessing and predicting the effects of therapeutic agents must be developed. The above observations resulted in the implementation of DNA direct sequencing as a clinical screening test for common EGFR mutations in patients with lung adenocarcinomas in many academic centers. This assay can be performed on fresh, frozen and archival formalin, fixed paraffin embedded (FFPE) tissue, including surgical resection specimens or fine needle biopsies. (24, 25) Even though microdissection is usually the first step in sample analysis, it is known that DNA direct sequencing may not detect mutations if tumor cells represent < 25% of the sample. Many other more sensitive mutation detection techniques have been reported including mutation-specific PCR assays, PCR with hybridization in real-time with mutation specific fluorescent probes, single-strand conformational polymorphism among others (26-29). As these techniques do not significantly improve diagnostic yield, direct sequencing is still considered the gold standard. The pathologist role in EGFR testing is to ensure that testing is performed only in adenocarcinomas or adenosquamous carcinoma, unless specifically requested by oncologist. Reports
indicate that mucinous type of BAC should not be tested for EGFR mutations. Pathologist should select
most cellular areas usually containing >50% of the tumor cells.

It is known that mutations in EGFR, KRAS, BRAF and HER2 genes are mutually exclusive in lung
adenocarcinoma. (30) KRAS mutations are predictor of failure
of EGFR TKI therapy. (31) They occur in adenocarcinomas of smokers and are adverse prognostic factor.
HER2 mutations are very similar to EGFR mutations, affecting adenocarcinomas with BAC morphology in
women, never smokers and may predict sensitivity to other targeted therapies. Therefore, clinical testing
in lung adenocarcinomas goes beyond EGFR mutation status, and some clinical laboratories are putting
into practice a comprehensive mutational profiles for lung adenocarcinoma since each of the above
mentioned mutations have some impact on treatment selection.

Despite the fact that most laboratories accepted direct sequencing or other mutational methods as most
reliable assays that predict good responders to TKI, there is still ongoing discussion about most
appropriate clinical testing for establishing EGFR status in lung adenocarcinoma, particularly gene copy
number analysis (FISH or CISH). (32-39) This is an area of much controversy in the field and
complicating factor is that EGFR mutations are frequently associated with increased EGFR gene copy
numbers. In the initial report by Cappuzzo et al. 33% of cases showed FISH + status defined as having
classical gene amplification (tight gene clusters and a ration of EGFR gene to chromosome of >2 or >15
copies per cell) and/or chromosome polysomy (>4 chromosomes in >40% of cells). These patients had a
higher response rate to gefitinib (36%) than FISH – patients (3%) and had a longer median survival (18.7
months vs. 7.0 months). Within the same cohort, 17% of cases were EGFR mutation positive, which was
associated with a response rate of 53%, compared to 5% in wild-type cases. Since this initial report,
several studies have compared EGFR gene copy number to EGFR mutations status with respect to their
relative predictive abilities. Some studies used previously defined criteria for EGFR FISH positivity and
others used qPCR. The results of the comparison analyses are varied and difficult to interpret. Cappuzzo
et al. also demonstrated that EGFR protein overexpression in 59% of tumors as assessed by
immunohistochemistry was associated with increased response (21% vs. 5%) and survival (11.5% vs.5.0
months). Both EGFR protein overexpression and EGFR mutations were associated with FISH+ status. As
with any other clinical test, technical considerations are important in assessing gene copy numbers and
protein expression. FISH can be readily performed on tissue sections, and the quantity and distribution of
hybridization signal is indicative of gene copy number. Scoring systems for increased ploidy are more
subjective, reflecting variable degrees of genomic instability rather than specific EGFR amplification.
In contrast, qPCR provides accurate quantification of copy number is not subject to interpretation variability,
but is affected by tumor purity within the analyzed sample.

EGFR protein quantification by immunohistochemistry is readily available using tissue sections, but
interpretation suffers from subjectivity and experimental conditions. All these observations resulted in
proposal for combined clinical testing for assessment of EGFR status in lung adenocarcinoma patients,
although recent evidence support EGFR mutation status as the most relevant marker for treatment
selection. (40, 41)

Unfortunately, almost all patients who respond to EGFR TKIs will develop resistance and may develop
recurrent disease resistant to further TKIs therapy. Acquired resistance to EGFR TKIs has been
associated with a second EGFR mutation in about 50% of cases, most frequently T790M in exon 20 of
the TK domain. (42) The frequency of this mutation may be underestimated particularly in cases with
EGFR amplification of the EGFR allele with the first mutation. Amplification of MET has recently been
identified as a second mechanism of EGFR TKI resistance. (43) Second generation EGFR TKIs are being
developed to target T790M and other mechanisms of acquired resistance to gefitinib and erlotinib. (44)

In summary, discovery of activating mutations in EGFR as a molecular basis for patient’s response to
EGFR TKI revolutionized molecular diagnostics of lung adenocarcinoma. EGFR mutation analysis is
currently accepted as the most accurate test for prediction of response to EGFR TKI. Combining EGFR
mutation analysis with EGFR FISH or CISH may provide additional useful information. Other genes
involved in lung adenocarcinoma carcinogenesis which may represent a new targets for therapies, most
likely will be tested in a similar way as EGFR in a near future.
REFERENCES


Universal MSI Testing in Colon Cancers
Larry Burgart, MD
Abbott Northwestern Hospital
University of Minnesota

Colon Cancer Molecular Pathways

- CIN - Majority of Cases
- Familial - FAP - 1%
  - 3rd decade
- Sporadic - LOH - 80%
  - 6th decade
  - APC, k-ras, DCC, p53

- MSI-H Minority of Cases
- Familial – Lynch S. - 2%
  - 5th decade
- Sporadic - MSI - 15%
  - 8th decade
  - DNA repair enzymes

Colorectal Carcinoma Relative Frequency by Pathogenesis

Relative Frequency

Sporadic APC/LOH (82%)
Sporadic DNA MMR (15%)
FAP (1%)
Lynch Syndrome (2%)

Microsatellite Instability

- Microsatellite: mono-dio-tetranucleotide repeat sequences through genome; polymorphic length
- LOH N T
- MSI N T

Defective DNA Mismatch Repair

Microsatellite Instability

DNA Errors DNA Replication

Mutations Repair Enzymes

Microsatellites

Pt 1: MSI-H
Pt 2: MSS

© 2008 College of American Pathologists. Materials are used with the permission of the faculty.
HNPCC vs. Lynch Syndrome

- HNPCC is clinically defined familial CRC
- Amsterdam Criteria
  1 - >3 relatives (>2 first degree) have histologically verified colorectal cancer
  2 - 2 successive generations affected
  3 - one of affected relatives <50 years old
- Approximately 60% of Amsterdam-type families are due to defective DNA mismatch repair ⇒ Lynch Syndrome (Bethesda conf)

Familial Influences in Colorectal Cancer

- FAP <1%
- HNPCC 2%
- Family history ~30%

Questions to address:

- Is it important to sort these 4 CRC categories?
- If yes, what’s the best approach?
  – What should pathologists do with CRC now?

Is MSI Status Clinically Important? (DNA Mismatch Repair Status)

- Improved Prognosis
  – Stage III MSI-H CRC behave like stage II MSS
  – Half as much mortality
  – Little, conflicting information on treatment influence

Samowitz, WS; Cancer Epidemiol Biomarkers Prev. 2001 Sep;10(9):917-23
Is MSI Status Clinically Important?  
(DNA Mismatch Repair Status)

- Prognostic YES
  - Stage III MSI-H CRC behave like stage II MSS
  - Half as much mortality
- Predictive NO – not yet anyway
  - In vivo suggests 5-FU based therapies don’t work
  - Conflicting data clinically
  - No rush to randomize stage III patients who are surviving at high rate to “no treatment”

Characteristic CRC Histology of Microsatellite Instability

<table>
<thead>
<tr>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sites: 46%</td>
<td>All sites: 94%</td>
</tr>
<tr>
<td>Proximal: 50%</td>
<td>Proximal: 96%</td>
</tr>
<tr>
<td>Distal: 17%</td>
<td>Distal: 92%</td>
</tr>
</tbody>
</table>

My clinicians are into prognostic stats. What test(s) should I do?

- Morphology
  - Architecture (50% sens, 95% spec)
  - TILs (80% sensitive, 80% specific)
Is MSI Status Clinically Important?  
(DNA Mismatch Repair Status)

- Improved Prognosis
  - Stage III MSI-H CRC behave like stage II MSS
  - Half as much morbidity
  - Little, conflicting information on treatment influence

- Lynch Syndrome Proband Identification
  - Influence screening/surgery choices
  - Sort at-risk family members

### Lynch Syndrome Target Population

- 2% of all colorectal carcinoma
- ~150,000 CRC in US each year
- ~3000 annual Lynch CRC
- Equal # of relatives with normal risk in high risk families

### Lynch Syndrome Risk Stratification

- Identify CRC (or related) cancer patient suspicious for Lynch (endometrium)
  - age, family history, histology
  - gastroenterologist, surgeon, geneticist, pathologist
- Perform phenotypic testing on cancer
  - microsatellite instability
  - immunohistochemistry

### Methods

- Microsatellite instability
  - "microdissect” CRC and nonneoplastic tissue
  - 5-10 microsatellite markers
  - MSI-H ≥30% markers unstable
- Immunohistochemistry
  - hMLH1, hMSH2, hMSH6, hPMS2
Lynch Syndrome Risk Stratification

- If positive phenotypic testing
  - risk of germline mutation depends on age, family history & which enzyme is deficient
  - strongly consider germline testing and kindred testing if germline positive
  - screen patient accordingly
- If negative phenotypic testing
  - screen accordingly
- Information is power

Lynch Syndrome Proband ID

- Open access – family or personal hx
- Reflexive testing
  - MSI testing on all CRC ≤50 years of age
  - Consider testing between 50-60 with characteristic morphology
  - Positive test (MSI-H) => genetic counseling, IHC, mutation analysis, family screening

Caveats

- Sort heritable vs. somatic MLH1 defects
  - Mutational analysis – much less than 100% sens
  - Methylation analysis – difficult, imperfect
  - BRAF mutation – very specific (+ sporadic)
    - Localized, easy to assay

Summary

- 15% CRC have defective DNA MMR
- 90% of those due to MLH1 methylation
  - MLH1 IHC (prognostic) or no testing
- Lynch Syndrome Proband ID
  - Open access => MSI and IHC
  - Reflexive (age) => MSI only
  - Sort heritable from somatic (IHC first)
    - BRAF & DNA MMR gene mutation analyses

Questions?
Colorectal carcinoma (CRC) was traditionally thought of as a monolithic disease process. During the last decade, however, variability in the underlying genetic pathogenesis has been described and has become clinically relevant. Colon cancer can now be split into (at least) 2 subsets based on molecular pathogenesis, the first characterized by chromosome instability and the second by genomic (genome-wide) instability. The chromosomal instability (CIN) pathway accounts for 80% to 85% of all colon cancers. The CIN pathway involves loss of tumor suppressor genes and activation of oncogenes, characteristically identified as loss of heterozygosity, and often manifests overt aneuploidy. Commonly involved genes include the adenomatous polyposis coli (APC) gene, K-ras, the deleted in colon cancer (DCC) locus and p53. This pathway also underlies familial adenomatous polyposis, which accounts for approximately 0.5% of all colon cancers.

Genomic instability results from a loss of DNA mismatch repair (MMR) activity. Normal DNA synthesis is associated with a low but finite error rate in terms of which nucleotide is put into the daughter strand. It is, of course, critical to have a high-fidelity repair system to maintain the integrity of the genetic code. One of these repair systems is a family of proteins that works together as the DNA MMR complex. These genes/proteins have unfortunate alphanumeric names, with the 4 major genes being MLH1, MSH2, PMS2 and MSH6. If any one of these proteins is absent, DNA MMR function is severely compromised. In the absence of DNA MMR activity, genome-wide mutations accrue over the course of several generations of cell division. DNA microsatellites (mononucleotide, dinucleotide, or tetranucleotide repeat sequences found throughout the genome) make excellent test sites to assay for genetic errors that occur as a result of defective DNA MMR. Accordingly, the standard molecular laboratory test for defective DNA MMR is amplification of several microsatellites to look for abnormal variation in length, which is referred to as microsatellite instability (MSI).

MICROSATELLITE INSTABILITY IS AN EXQUISITELY SENSITIVE AND SPECIFIC MARKER FOR DEFECTIVE DNA MMR

Defective DNA MMR accounts for 15% to 20% of sporadic colon cancers, which typically occur at an older age than sporadic CIN carcinomas, are more frequently present in the proximal colon, and affect women twice as often as men. Lynch syndrome is a familial syndrome due to heritable defective DNA MMR that accounts for 2% of all colon cancers, demonstrates autosomal-dominant inheritance, has no gender bias, and has a broad age distribution with peak in the fifth decade. Sporadic colon cancer with defective DNA MMR is essentially always due to the same, very specific DNA alteration. All of these sporadic MSI-positive CRCs result from methylation of the MLH1 gene promoter, which stops transcription of both MLH1 alleles. Therefore, not only do all sporadic CRCs due to defective DNA MMR have MSI but, they also are essentially all due to lack of MLH1 expression.

Based on the above discussion, CRCs can be divided into 4 subtypes: (1) familial colon cancer due to chromosomal stability (familial adenomatous polyposis), (2) sporadic colon cancers due to CIN (ordinary-type colon cancer), (3) familial colon cancer due to defective DNA MMR (Lynch syndrome), and (4) sporadic colon cancer due to defective DNA MMR.

IS IT IMPORTANT TO IDENTIFY SPORADIC CRC DUE TO DEFECTIVE DNA MMR?

Sporadic colon cancers with defective DNA MMR have a significantly improved prognosis over CIN-pathway CRCs. In fact, stage III CRCs with defective DNA MMR have a survival curve superimposable on that of stage II CIN cancers. This equates to half as much mortality in stage III CRC with defective DNA MMR compared to stage II CRC with CIN. Unfortunately, there are few data on whether this is
dependent or independent of treatment, and the available data are conflicting. Currently, treatment decisions are not made based on DNA MMR status, despite in vitro data suggesting that 5-FU–based therapies do not work in these cancers. However, as chemotherapeutic regimens become refined and as panels of prognostic markers are put in place to determine which stage II and III CRC patients should receive therapy, these data will become essential.

IF MY CLINICIANS ARE INTERESTED IN THIS PROGNOSTIC INFORMATION, WHAT IS THE BEST TEST TO PERFORM?

The candidate tests for detection of defective DNA MMR in colon cancer include histology, immunohistochemistry, and molecular MSI testing. Characteristic histologic features do exist for CRC with defective DNA MMR. The most specific morphologic feature is cribriform/solid (medullary) architecture without anaplastic cytology. Mucinous colon cancers (colloid and signet ring) also have an increased incidence of defective DNA MMR, but are much less specific than the medullary pattern. The suboptimal aspect of using the cribriform/solid architectural pattern as a test for defective DNA MMR is poor sensitivity. The sensitivity is approximately 50%; half of all CRCs with defective DNA MMR have ordinary-type morphology with gland formation and desmoplasia.

Tumor-infiltrating lymphocytes are more sensitive for the detection of defective DNA MMR. Tumor infiltrating lymphocytes are defined by their intimate association with colon cancer cells. This does not include peritumoral or Crohn-like inflammation. Unfortunately, there is a sensitivity-specificity trade-off, which results in approximately 80% sensitivity with 80% specificity when approximately 5 tumor-infiltrating lymphocytes per high-power field are present within a CRC. Therefore, this is a good but not perfect marker for the detection of MSI-positive colon cancers. For sporadic CRC with defective DNA MMR, immunohistochemistry is an excellent assay. MLH1 immunoperoxidase staining has essentially 100% sensitivity and specificity for defective DNA MMR in the sporadic setting due to the homogeneous underlying molecular pathogenesis (MLH1 promoter methylation). One would have to do this immunostain on all colon cancers to have 100% sensitivity, but molecular microsatellite analysis would be redundant and therefore unnecessary. Immunohistochemical reagents are available for MLH1, MSH2, MSH6, and PMS2 expression. The vast majority of all cell types (except those very terminally differentiated) normally demonstrate nuclear expression of each of these DNA MMR enzymes. This provides an extremely useful internal positive control when assaying for aberrant loss of a DNA MMR protein, as any benign cells - stromal or epithelial - will express the DNA MMR proteins.

IS IT IMPORTANT TO PERFORM IMMUNOHISTOCHEMISTRY AND/OR MOLECULAR MICROSATELLITE ANALYSIS TO DETECT LYNCH SYNDROME PROBANDS?

Approximately 2% of the 150 000 CRCs diagnosed in the United States each year occur in patients with Lynch syndrome (Hereditary Nonpolyposis Colorectal Cancer Syndrome due to defective DNA MMR). This means there are approximately 3000 CRCs per year in Lynch syndrome families. In addition, Lynch syndrome families are at risk for endometrial, upper gastrointestinal, upper urinary tract, ovarian, and pancreaticobiliary carcinomas. Identifying these families allows for sorting of at-risk family members. This autosomal-dominant disease results in 50% of family members having an 80% to 100% lifetime risk of carcinoma development, with the other half having normal cancer risk. Therefore, identification of Lynch syndrome probands can tremendously impact decision-making (e.g., screening, prophylactic surgery).

It is reasonable to clarify some terminology at this point. Hereditary nonpolyposis colorectal cancer syndrome (HNPCC) is a clinically defined term based on strong family history of colon cancer. The original HNPCC definition, referred to as the Amsterdam Criteria, includes the following: (1) 3 involved relatives, 2 of whom are first-degree relatives; (2) successive generations involved; and (3) 1 involved patient younger than 50 years. Even gold-plated families such as these have an incidence of heritable DNA MMR of 60%, with the remaining 40% of HNPCC families unexplained as to their genetic basis. Disease in those HNPCC families with heritable DNA MMR is referred to as Lynch syndrome. Approximately 30% of colon cancer patients have at least 1 first- or second-degree relative also with colon cancer.
Immuno-histochemical findings in Lynch syndrome cases are much more complex than in sporadic MSI-positive CRCs. While 100% of Lynch syndrome-related carcinomas have MSI, only approximately 95% of them will have abnormal immuno-histochemical findings (loss of expression of a DNA MMR enzyme). The rough distribution of abnormal DNA MMR gene and subsequent loss of protein expression within Lynch syndrome families is as follows: MLH1, 40%; MSH2, 40%; MSH6, 5%; and PMS2, 5%. A small percentage of Lynch syndrome families develop MSI-positive carcinomas with intact staining of all 4 of these enzymes due primarily to a missense mutation affecting function but not antigenicity, or less commonly involvement of another minor protein in the complex.

Based on the above information, recommended testing for Lynch syndrome includes a combination of molecular microsatellite analysis and immuno-histochemistry for MLH1, MSH2, PMS2, and MSH6. This allows for high sensitivity detection of MSI within the tumors and identification of which enzyme is abnormal within that family, in turn guiding the subsequent germline mutation analysis. Both microsatellite analysis and immuno-histochemistry are performed on paraffin-embedded tissue. The microsatellite analysis requires both tumoral and non-tumoral tissue for comparison.

**WHICH CRC PATIENTS SHOULD BE TESTED FOR LYNCH SYNDROME?**

The rule of thumb for Lynch Syndrome testing includes any patient with CRC arising with positive family history and/or young age. A series of National Institutes of Health–sponsored consensus conferences have established the Bethesda Guidelines for selecting patients to test. There are instances in which it can be difficult to differentiate between familial and sporadic defective DNA MMR colon cancer. For example, a 50-year-old with a colon cancer showing high levels of MSI and loss of MLH1 expression can be ambiguous as to germline versus somatic abnormality if the family history is not clear or unavailable. Germline analysis is helpful in these situations, but is also less than 100% sensitive. Recently, a specific B-raf mutation in codon 600 has been identified in a majority of sporadic (methylated) defective DNA mismatch repair CRC cases, which rarely is present in Lynch syndrome carcinomas. Therefore, B-raf mutational analysis is a useful part of the testing algorithm in this situation.

In summary, 15% to 20% of CRCs are due to defective DNA MMR. Ninety percent of these cases will be caused by silencing of MLH1 expression due to methylation of the gene promoter. These sporadic CRCs with defective DNA MMR have a significantly improved prognosis, but therapy is unchanged. Lynch syndrome proband identification is a very active testing program taking advantage of both microsatellite analysis and immuno-histochemistry on paraffin-embedded tissue with subsequent gene mutation analysis (peripheral blood DNA) to sort at-risk family members.

**References**


Universal MSI Testing in Colon Cancers
Lawrence J. Burgart, MD


Universal MSI Testing in Colon Cancers
Lawrence J. Burgart, MD


Translocation Analysis in Prostate Cancer

Mark A. Rubin, MD
Vice Chair of Experimental Pathology
Professor, Dept. of Pathology and Laboratory Medicine
Weill Cornell Medical College
www.Rubinlab.org

Disclosure

The University of Michigan and the Brigham and Women's Hospital have filed a patent on ETS gene rearrangements. Drs. Chinnaiyan, Tomlins, Perner, Demichelis, Rubin, and others are co-inventors, and the diagnostic field of use has been licensed to Gen-Probe Incorporated.

Learning Objectives

• What are the critical gaps in prostate cancer biomarkers for clinical use?
• What is the significance of gene fusion cancers clinically?
• What to we know about TMPRSS2-ETS prostate cancer in March 2008?
• What is the possible future clinical utility of exploiting this common gene fusion?

Agenda

• Prostate cancer and PSA: the dilemma
• CML, Burkitt Lymphoma, and Prostate cancer: role of gene fusion in cancer
• TMPRSS2-ERG prostate cancer is the most common gene fusion described to date
• Clinical implications for the future

Critical Questions for Prostate Cancer Biomarker Development
2006 Estimated US Cancer Cases*

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>720,280</td>
<td>679,510</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>31%</td>
<td>Breast</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>13%</td>
<td>+12%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>10%</td>
<td>+11%</td>
</tr>
<tr>
<td>Melanoma of skin</td>
<td>6%</td>
<td>+6%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>5%</td>
<td>+4%</td>
</tr>
<tr>
<td>Kidney</td>
<td>3%</td>
<td>Thyroid</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>3%</td>
<td>+3%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>3%</td>
<td>Ovary</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2%</td>
<td>+2%</td>
</tr>
<tr>
<td>All Other Sites</td>
<td>18%</td>
<td>+2%</td>
</tr>
</tbody>
</table>

Source: American Cancer Society, 2006.

Europe and American PCA Incidence and Mortality

695 Patients Selected for Trial

- Watchful Waiting: 348
- Radical Prostatectomy: 347
- Lymph node positive: No treatment: 23
- Non-compliant with randomization: 30 WW, 31 RP
A Preoperative Nomogram for Disease Recurrence Following Radical Prostatectomy for Prostate Cancer

Michael W. Kattan, James A. Eastham, Alan M. Stephenson, Thomas M. Wheeler, Peter T. Scardino

Background: Five published studies have combined clinical prognosis factors into risk profiles that can be used to predict the likelihood of recurrence or metastatic progression in patients following treatment of prostate cancer. We developed a nomogram that allows prediction of disease recurrence using only preoperative clinical factors in patients with clinically localized prostate cancer who have been treated with radical prostatectomy.

The Three Prostate Cancers

Lethal Disease
No Cure Possible
Benefit from Treatment
Do well regardless of Treatment
Can we capture a molecular signature to represent this?

RESEARCH ARTICLE

Recurrent Fusion of TMPRSS2 and ETS Transcription Factor Genes in Prostate Cancer


Recurrent chromosomal rearrangements have been strongly correlated with prostate cancer development. We used a high-resolution approach to determine the breakpoints and structural elements in the majority of prostate cancer samples harboring TMPRSS2-ETS fusions and ETV1 fusions. The genes are predominantly located in the TMPRSS2 locus, indicating that these fusion genes are the result of gene amplification. The data suggest that disruption of the ETS transcription factors results in transcriptional activation of TMPRSS2 and ETV1. Further, the data indicate that TMPRSS2 and ETV1 fusions are mutually exclusive. These findings provide new insights into the molecular mechanisms that underlie prostate cancer development.
Value Added Diagnostics: Emerging Tests in Clinical Practice

Overview

What role do recurrent gene fusions play in the development of cancer?

Chronic Myeloid Leukemia (CML): a gene fusion cancer

Impact of gene fusions on cancer causation

Burkitt Lymphoma

- IgH Chain gene
- MYC

CML

- BCR gene
- BCR-ABL1

Chromosomal Aberrations in Cancer

- Simple and disease-specific
- Complex and disease-specific
- Acute leukemia
- Myelodysplastic syndromes
- Multiple myeloma
- Leukemoid reactions
- Malignant lymphomas
- Leukemia/lymphoma
- Mesenchymal tumors
- Epithelial tumors

Mitelman, F., Mutation Research 462:247

Linear Progression Model of Somatic Alterations

De Marzo et al. NEJM

Translocations

- Less than 1% of described translocations occur in epithelial cancers
  - ETV6-NTRK3 in secretory breast cancer
  - RET and NTRK1 with multiple partners in thyroid papillary carcinoma
  - PAX8:PPARγ in thyroid follicular carcinoma
  - PRCC:TFE3 in renal papillary carcinoma
- Recurrent translocations have not been described in common epithelial cancers such as colon, breast, lung and prostate adenocarcinoma

Why have so few gene rearrangements been characterized in carcinomas?

- Molecular basis of carcinomas is due to genomic instability leading to mutations, deletions and amplifications
- Many epithelial cancers, including prostate carcinomas are difficult to culture and karyotype

Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer

Felix Mitelman, Bertil Johansson & Fredrik Martens

Nature Genetics 36, 331 - 334 (01 Apr 2004)

Estimated cancer mortality distribution by type and fusion status

20% mortality due to fusion cancers
94% Carcinomas with fusion TMPRSS2-ETS

Gene fusion involved in cancer initiation: What is the evidence?

1. Fusion associated with tumor phenotype
2. Distinct molecular signature
3. Treatment of chimera eradicates tumor (e.g., CML)
4. Animal models recapitulate human tumor
5. In vitro silencing of fusion lead to decreased tumorigenicity


Heterogeneity of Prostate Cancer: Version October 2007

Localized PCA:
48.5% (115/237) TMPRSS2-ERG fusion positive
30% (71/237) TMPRSS2-ERG fusion through deletion
18.5% (44/237) TMPRSS2-ERG fusion through translocation

Independent Confirmation by multiple groups as of Oct 2007

- PSA screened surgical populations
- RT-PCR evaluation for fusion
- TMPRSS2-ERG predominant fusion

© 2008 College of American Pathologists. Materials are used with the permission of the faculty.
FUSION STATUS
Cumulative Incidence Ratio: 2.7 (p-value=0.01, 95%CI=(1.3, 5.8))

To account for competing causes of death over time cumulative incidence regression 
(Kalbfleisch et al. 2002) implemented in cprsk R library.

Demichelis F, Fall K et al., Oncogene

• 33% of 405 T1-T3 cases with Fusion
• 17% of 133 T1 cases with Fusion
• Fusion and Gleason score associated
• Fusion through deletion associated with cancer specific death (HR=1.72, 95% CI 10.2-2.89, p=0.042) after correcting for baseline PSA and Gleason score.
100% FUSION NEGATIVE
- Normal Prostatic Tissue (47)
- Benign Prostatic Hyperplasia (15)
- Atrophy (9) (unspecified)
- Simple Atrophy (17)
- Simple Atrophy with Cyst Formation (6)
- Post Atrophic Hyperplasia (6)

Perner et al., AJSP 2007

HGPIN:
- 81% (21/26) Fusion negative
- 19% (5/26) Fusion through deletion
- 0% (0/26) Fusion through translocation

Most of fusion positive PIN lesions in tight proximity of PCA and all have the same fusion pattern as the corresponding PCA.

Morphologic features of TMPRSS2-ERG prostate cancer

A. PCA showing blue mucin. Adjacent normal glands. B. FISH highlights the TMPRSS2-ERG through deletion status of this PCA.
C. Cribriform pattern of PCA. D. FISH highlights the TMPRSS2-ERG through deletion status of this PCA.

Mosquera et al., J Pathology 2007

Early Detection Research Network (EDRN)
Prostate Biopsy Study: Michigan and Harvard
100 Needle Biopsies with Prostate Cancer analyzed for TMPRSS2-ERG

46% TMPRSS2-ERG Fusion
- 63% (29/46) through deletion
- 37% (17/46) through incertion

No association with Gleason score

Morphology associated with TMPRSS2-ERG Fusion
- Cribriform growth pattern (p=0.03)
- Blue-tinged mucin (p=0.01)
- Macronucleoli (p=0.02)
- Collagenous micronodules (4/4 F+)

Oct 2007
Rubin - 9

Diagnosis and therapeutic implications

**CML**
- Only one fusion
- RTK (BCR-ABL1)
- Chimera target for inhibitors
- Fusion diagnostic 
  (highly specific)
- +/- Prognosis

**Fusion PCA**
- Multiple fusions
- Transcription Factors 
  (e.g., ETS genes)
- TF difficult drug targets
- Fusion diagnostic 
  (highly specific)
- +/- Prognosis
Summary

1. **TMPRSS2-ETS** PCA is the most common translocation described.
2. Fusion product is hormonally regulated (tmprss2)
3. **TMPRSS2:ERG** early molecular event
4. **TMPRSS2:ERG** PCA associated with phenotypic alterations
5. **TMPRSS2:ERG** may confer a more aggressive clinical course in the absence of clinical treatment (implications for local treatment)
6. Urine-based fusion assay highly specific for PCA
7. FISH based diagnostic/prognostic test vulnerable to heterogeneity

References

All literature related to gene fusion prostate cancer is available at:

[www.genefusionpca.org](http://www.genefusionpca.org)
DEVELOPMENT OF DIAGNOSTIC TESTS FOR TMPRSS2-ERG PROSTATE CANCER

Prostate cancer (PCA) is a common and clinically complex disease with marked variability in disease progression with an anticipated 219,000 new cases to be diagnosed this year in the United States and 27,000 deaths expected from the disease. Despite its widespread use, serum Prostate-Specific Antigen (PSA) has limited accuracy as a PCA biomarker, and new biomarkers are needed to improve early detection of aggressive PCAs. Novel bioinformatics approaches have recently lead to a striking discovery indicating that 50% of men diagnosed with PCA harbor an acquired chromosomal translocation that results in the fusion of the promoter region of the Transmembrane protease, serine 2 (TMPRSS2) gene to the coding region of members of the erythroblast transformation specific (ETS) family of transcription factors, most commonly V-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG). PCA with the rearranged genes have a different biologic logic when compared to PCA without the translocation and has been associated with a poorer prognosis. Recent studies have reported variations in the structure of the gene fusions in PCA yielding different fusion transcript isoforms which has been already noted for other fusions involving the ETS family in Ewing sarcoma (ES). Eighty-five percent of ES cases harbor a t(11;22)(q24;q12) translocation resulting in a fusion protein comprising the amino terminal part of the EWS gene and the ETS-DNA binding domain of the FLI1gene. Eighteen possible types of in-frame EWS-FLI1 transcript variants have been described which reflects genomic breaks in 1 of 4 EWS introns and 1 of 6 FLI1 introns. Despite this heterogeneity, many studies have lead to the observation that 2 major chimeric fusion types account for most of the EWS-FLI1 positive ES, one of which is associated with a more favorable prognosis. In PCA TMPRSS2-ERG fusion variants can be divided into 2 broad groups: one that contains the first exon of the TMPRSS2 gene juxtaposed to different exons of the ERG gene; and the other that contains the first 2 or 3 exons of TMPRSS2 juxtaposed to different exons of the ERG gene. Interestingly, one isoform from the latter group starts from the ATG of the TMPRSS2 gene (exon 2) and includes the first 5 amino acids of the TMPRSS2 protein, is in frame with ERG exon 4 but is missing the first 12 amino acids of the ERG protein. Though based on a small sample population this isoform was associated with a more aggressive phenotype. To date, there have been no reports characterizing the expression of the different isoform in a large sample populations.

Clinical Implications

Urine Based Assay: Over diagnosis is substantial in patients diagnosed with clinically localized PCA and has reached alarming proportions for cancers detected by PSA-testing which may in part be explained by changing biopsy practice patterns over the last decade. Reliable distinction between PCA patients who do not require aggressive treatment because they have indolent disease from those patients who cannot be cured by local treatment alone would have significant impact on clinical practice. Over treatment would be reduced and expensive systemic treatment would be better targeted to those who need it using the least invasive procedure possible. Clinical and pathology parameters alone have not successfully made this distinction. The recently described TMPRSS2-ETS rearrangements in PCA and associated molecular events hold great promise in providing additional information beyond conventional clinical and pathological parameters. Moreover, emerging evidence suggests that the expression of specific TMPRSS2-ERG transcript variants is associated with disease progression. Therefore, the first important clinical diagnostic test will be the development of a highly sensitive and specific, urine-based assay to detect PCA that will potentially determine risk. The talk will focus on efforts to develop a clinical test for the urine-based detection of TMPRSS2-ERG transcripts (mRNA) and some of the challenges due to the diversity of fusion isoforms.
Tissue-Based FISH Assays: Recent work suggests that TMPRSS2-ERG fusion prostate cancer is a distinct molecular subclass (Setlur, unpublished observations) associated with prostate cancer progression. We have described previously the FISH assay for the translocation of TMPRSS2-ERG. Briefly, because TMPRSS2 and ERG are so close together on chromosome 21, a break-apart probe system was used. This identifies when the telomeric and centromeric components of ERG split apart and is indirect evidence of TMPRSS2-ERG fusion. With this system, a nucleus without ERG rearrangement demonstrates 2 pairs of juxtaposed red and green signals, usually forming a yellow merged signal. A nucleus with an ERG break-apart (i.e. TMPRSS2-ERG fusion through insertion) shows splitting apart of the red and green signal for the translocated ERG allele and maintenance of a juxtaposed red-green (yellow) signal pair for the non-translocated ERG allele. A nucleus with TMPRSS2-ERG fusion through deletion shows loss of one green signal with preservation of the associated red signal and a juxtaposed red-green (yellow) signal pair for the non-translocated ERG allele. The utility of the FISH assay may serve as the gold standard to diagnosis TMPRSS2-ERG fusion prostate cancer. This information will be used for diagnosis and prognosis. Potentially if drug targets exist establishing fusion status will be important for treatment decisions. Making the definitive diagnosis of a fusion prostate cancer will also have to take into account the inherent heterogeneity of prostate cancer. In two recent studies, the heterogeneity of TMPRSS2-ERG PCA was documented by examining discrete tumor nodules from the same prostate gland. Within a tumor nodule, homogeneity with respect to the fusion event was the rule. Almost all neoplastic cells within a specific discrete nodule demonstrated fusion or the absence of fusion. However, between nodules from the same patient diversity of fusion status was observed. Therefore, a highly specific assay such as the urine-based assay may be needed to screen for fusion status. Alternatively, sampling from all lesions of a prostatectomy gland would be needed.

References

4. F. Demichelis, K. Fall, S. Perner et al., Oncogene 26 (31), 4596 (2007).
17. R. Mehra, B. Han, S. A. Tomlins et al., Cancer Res 67 (17), 7991 (2007).
Value Added Diagnostics: Emerging Tests in Clinical Practice

Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD

Disclosure
• Dr. Asa has the following disclosures:
  – She is a consultant to Arius Research, Toronto; she assists in the pathologic assessment of mouse xenografts to determine the effects of monoclonal antibodies as cancer therapies
  – She is an unpaid member of the Pathologist Advisory Committee to Cerner Corp., since her department is a large user of the CoPath LIS.

Learning Objectives
• To understand the molecular alterations in thyroid cancer of follicular cell derivation
• To identify molecular markers that can assist in the diagnosis, prognosis and management of thyroid nodules
• To recognize techniques and pitfalls in the application of molecular tests for thyroid cancer

Thyroid Histology: “Gold Standard”

Hyperplasia vs. Neoplasia
Benign vs. Malignant
Indolent vs. Aggressive Malignancy
• Observer-dependent
• Inconsistent
• Lack scientific criteria

Agenda
• Clonality: hyperplasia vs. neoplasia
• Genetic alterations in signaling pathways
• Genetic alterations in differentiation factors
• Genetic alterations in oncocytic tumors
• Growth factors in thyroid cancer
• Cell cycle regulation
• Adhesion molecules
• Biomarkers and clinical applications

Clonality

Hyperplasia vs. Neoplasia
Polycional vs. Monoclonal
Reactive vs. Autonomous
Sporadic Nodular Goiter

- Multinodular “colloid” goiter
- Occasionally associated with hyperthyroidism – “Plummer’s disease”
- Etiology and pathogenesis NOT understood

Clonality in Nodular Goiter

- Dominant nodules often monoclonal
- Nodules may show LOH or aberrant methylation
- Multiple nodules from a single goiter exhibit activation of the same allele

Diagnostic criteria


Clonality Studies: Multifocal Papillary Carcinoma

- Using X-chromosome inactivation or ret/PTC rearrangements
- Majority of multifocal lesions are different clones, only minority have similar profiles
- Proves that at least the majority of these are separate lesions rather than intrathyroidal dissemination of a single tumor

Follicular Adenomas with Papillary Architecture

- “Papillary adenomas”
- Monoclonal benign neoplasms
- Activating mutations of TSH-receptor or Gsα
- Plummer’s disease

G-Protein Activation

\[
\begin{align*}
\text{OFF} & : \text{Receptor GDP} \xrightarrow{\text{Ligand}} \text{GTP} \\
\text{ON} & : \text{Receptor GDP} \rightarrow \text{GTP} \\
\end{align*}
\]

Ret/PTC Rearrangements

- Chromosomal rearrangement involving chromosome 10 ret
- Fusion of the ret tyrosine kinase to:
  - \(CCDC6\ (H4) = \text{ret/PTC1}^*\)
  - \(R1\alpha = \text{ret/PTC2}\)
  - \(NcoA4\ (ele) = \text{ret/PTC3}^*\)
- At least 15 identified to date
Ret/PTC Rearrangements

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>TM</th>
<th>TK</th>
</tr>
</thead>
<tbody>
<tr>
<td>ret</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ret/PTC-1</td>
<td>CCDC6 (H4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ret/PTC-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ret/PTC-3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These rearrangements result in cytoplasmic protein; antibodies against ret identify the C terminus that is conserved.

Ret/PTC-1

- First molecular alteration found in thyroid cancer
- Alters nuclear envelope and chromatin structure to account for nuclear features of papillary carcinoma

Methods of Ret/PTC Analysis

- DNA
  - PCR analysis difficult due to variable break-point sites leading to heterogeneous tumor profiles
- RNA
  - RT-PCR for ret/PTC mRNA is the "gold standard"
  - Variability of expression; not "all or none"
- Protein
  - Immunohistochemistry using antisera to C terminus
- FISH
  - Not widely available but promising

Ret Immunohistochemistry

Ret/PTC Rearrangements in PTC: Significance

- Diagnostic marker of papillary carcinoma
- High levels of ret/PTC expression may predict aggressive lymphatic involvement
  (Sugg et al., JCEM 1996;81(9):3360-5)
- No correlation of ret/PTC expression with structural features of aggressive behavior
  - except possibly ret/PTC-3 in solid variant
  - (Nikiforov et al., Am J Surg Pathol 2001;25:1478-84)

NTRK1 Rearrangements

- NTRK1 (TRK, TRKA) on 1q22
- Transmembrane tyrosine kinase receptor for nerve growth factor
- NTRK1 rearrangements with ectopic expression and constitutive activation of the tyrosine kinase analogous to RET rearrangements
- 5-13% of sporadic and 3% of post-Chernobyl childhood PTCS
- TPM3, TPR, and TFG fusion partners forming TRK-T1 and T2, TRK-T3
BRAF Mutations

- Most common genetic event in thyroid cancer
- Diagnostic marker of PTC
- Genotype-phenotype correlations
  - BRAF V600E in classical variant PTC (common)
  - BRAF K601E in FVPTC (rare)
  - VK600-1E deletion (BRAF VK600-1E) in solid variant (single case)
- Prognostic significance controversial

Ras Mutations

- Mutations involving codon 61 of HRAS and NRAS
- Correlation with follicular morphology
- Variable experience in benign and WDTC
  - Rare in WDTC and predict aggressive behavior
- High incidence in insular carcinoma

Alterations in Differentiation Factors

- Pax 8- PPARγ Fusions
  - Pax 8, a transcription factor that regulates expression of thyroglobulin, thyroid peroxidase and NaI symporter
  - PPARγ, a transcription factor implicated in cell growth and differentiation
- In-frame rearrangement results in fusion protein that interferes with function of both

Pax 8-PPARγ 1 Fusion Oncogene

t(2;3)(q13;p25)

- Diagnostically applicable by FISH, or immunohistochemistry for PPARγ

Alterations in Oncocytic Tumors

- Hürthle cell adenoma, Hürthle cell carcinoma
  - distinguished by invasive behavior
  - controversial because of unpredictable behavior
- Hürthle cell papillary carcinoma
  - defined by papillary architecture
Hürthle Cell Papillary Carcinoma

Is There a Follicular Variant Hürthle Cell Papillary Carcinoma?

Representative RT-PCR for ret/PTC-1,2,3 in Hürthle Cell Tumors

Molecular Basis of Hürthle Cell Papillary Carcinoma

mtDNA, GRIM19

Cell Cycle Regulation

• ret/PTC identifies Hürthle cell tumors that have lymph node metastases
  – allows distinction from Hürthle cell adenoma
  – better prognosis than Hürthle cell carcinoma

• mtDNA somatic events
• Mutations in non-neoplastic and neoplastic oncocytes cells
  – Not specific to neoplastic transformation
  – Associated with BRAF, ret/PTC etc.
• GRIM19 (19p13.2) somatic and germline events

• Under-expression in papillary thyroid carcinoma with metastasis and undifferentiated thyroid carcinoma
• Over-expression in papillary thyroid carcinoma with metastasis and undifferentiated thyroid carcinoma

(Cheung et al, J Clin Endocrinol Metab 85: 878-882, 2000)


© 2008 College of American Pathologists. Materials are used with the permission of the faculty.
Cyclin D1 and p27 Expression Predict Metastasis in PTC

Khoo et al, JCEM 2002; 87:1810-3,1814-18; Arch Otolaryngol 2002; 128:253

p53 Alterations

Mutations are common in Anaplastic carcinoma ↓

• Immunolocalization correlates with extent of disease, extrathyroidal involvement, recurrence and poor outcome in differentiated carcinoma

(Hosai et al, Endocr Pathol 1997, 8:21-28)

Cadherins are Downregulated

• E-cadherin (CDH1, 16q22) is highly expressed in normal thyroid, FAs, and minimally invasive FCs
• Low or absent in widely invasive, recurrent, metastatic and undifferentiated thyroid carcinoma
• Somatic mutations of CDH1 are infrequent
• Downregulation is by epigenetic silencing (methylation) of the CDH1 promoter

Soares et al Int J Cancer 1997; 70(1):32-38

β-Catenin Mutations

• Identified in poorly differentiated ("insular") carcinoma
• Reduced membrane immunolocalization in all tumors, correlates with aggressiveness
• Nuclear translocation due to exon 3 mutation in about 25% of insular carcinomas and in 65% of undifferentiated carcinomas

(Garcia-Rostan et al, Am J Pathol 2001;158:987)

Microarrays of Thyroid Cancer

• CITED-1 (L)
• Galectin-3
• Fibronectin (R)
• HGF, MET
• TPO
• COX-2
• CD44V6
• CD57

Prasad et al, Modern Pathology 2005;18:46

Fibronectin is Upregulated in Papillary Thyroid Carcinoma

• Increased cDNA expression in microarray studies of papillary carcinoma of normal
• Diminished FN immunoreactivity reported at invading edge of aggressive thyroid cancers
• Negative in poorly-differentiated and anaplastic carcinomas
• Function unclear
  – Increasing invasion?
  – Reactive upregulation?
Fibronectin in Thyroid Cancer

- Fibronectin mediates adhesion in thyroid carcinoma and restrains tumour growth
- VD upregulates fibronectin and restores adhesiveness of thyroid carcinoma
- The PTEN/PI3 Kinase pathway is involved in FN regulation and VD action on FN and adhesion
- The mechanism underlying overexpression in papillary carcinoma is unclear but appears to be compensatory

(Liu et al, Molecular Endocrinology 2005; 19(9):2349-57)

CEACAM1

aka biliary glycoprotein (BGP), CD66a, C-CAM1 and pp120

- A member of the CEA family (Ig superfamily)
- A putative TSG
  - Down-regulated in colon, prostate, liver, endometrial, bladder and breast cancer
  - Reduces proliferation in human prostate cancer cell lines in vitro and in vivo
- Also implicated as an oncogene
  - Over-expressed in gastric cancer, non-small cell lung cancer and malignant melanomas
  - Facilitates metastatic tumor spread
  - Shows angiogenic function as a major target of VEGF

CEACAM1 in Thyroid Cancer

- CEACAM1 is expressed in a small thyroid malignancies with lymph node spread
- CEACAM1 has a novel dual role in thyroid carcinoma: it has a suppressive effect on thyroid cell proliferation and increases adhesion, while promoting invasion and metastasis

(Liu et al, Oncogene 26:2747-58, 2007)

Biomarkers and Thyroid Cancer

- CITED-1 (L)
- Galectin-3
- Fibronectin (R)
- HGF, MET
- TPO
- COX-2
- CD44V6
- CD57

Prasad et al. Modern Pathology 2005;18:48

Epigenetic Dysregulation

- Unknown: HBME-1
- Cell structure: CK19, galectin-3
- Differentiation markers: TPO
- Cell cycle regulators: p27, cyclin D1
- Adhesion molecules: Fibronectin, CEACAM1
Markers of Malignancy:
HBME-1, Galectin-3

Markers of Papillary Carcinoma: CK19

- One of many keratins
- Identified diffusely in 60% of papillary carcinomas
- Also seen in reactive nontumorous thyroid

Molecular Studies:
Progression in Thyroid Cancer

Summary

- Diagnostic markers of thyroid cancer
  - BRAF - ras
  - Ret/PTC - p53
  - HBME-1 - β-catenin

- Prognostic markers in thyroid cancer
  - p27 - Pax8-PPARγ
  - Cyclin D1 - p53
  - FN - CEACAM1

Pearls of Pathology

- Make sure your technology is sensitive (but not too much so) and specific
- What you can do in surgical pathology you can often do in cytology
  Cheung et al JCEM 2001; 86(5):2187-2190
  Salvatore et al JCEM 2004; 89:5175-5180
- Science can be helpful where there is controversy, but some people will take longer to believe

Questions?
Abstract

Thyroid cancer is increasing in incidence and its diagnosis is difficult and often controversial. Recent advances have improved our understanding of its pathogenesis and we now recognize genetic alterations that activate a common effector pathway involving the RET/RAS/BRAF signaling cascade as a hallmark of differentiated carcinomas of follicular cell derivation. Some of these changes have been associated with radiation exposure as a pathogenetic mechanism. Defects in transcriptional and post-transcriptional regulation of adhesion molecules and cell cycle control elements are thought to modify the biological behavior of thyroid cancer. Several of the genetic mutations and rearrangements associated with this cancer provide powerful ancillary diagnostic tools and can also be used to identify new therapeutic targets.

Objectives

- To understand the molecular alterations in thyroid cancer of follicular cell derivation
- To identify molecular markers that can assist in the diagnosis, prognosis and management of thyroid nodules
- To recognize techniques and pitfalls in the application of molecular tests for thyroid cancer
Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD

- Introduction

Thyroid nodules may be hyperplastic, benign adenomas or malignant lesions derived from thyroid follicular epithelial cells or C cells. Thyroid cancer is the most common malignancy of endocrine organs and incidence rates have steadily increased in recent decades(1;2). More than 90% of thyroid carcinomas are derived from follicular cells. The majority of these are well-differentiated papillary thyroid carcinomas (PTCs). Although initially defined by architectural criteria, the histologic diagnosis of PTC relies on nuclear features that predict the propensity to lymphatic spread(3). The diagnosis of this most frequent thyroid malignancy (85-90%) has been increasing, possibly due to changing recognition of nuclear criteria or possibly due to increasing dietary iodine supplementation. Follicular carcinoma (FC) is characterized by hematogenous spread and the frequency of its diagnosis has been declining in most parts of the world with iodine-sufficient diets(4). Most well differentiated thyroid cancers behave in an indolent manner, are cured by surgery with or without radioactive iodine therapy, and have an excellent prognosis. However, a subset metastasizes to lung, bones, brain, and liver, sites that are not often amenable to surgical excision or radioactive iodine therapy. Poorly-differentiated and undifferentiated thyroid carcinomas are also highly aggressive, lethal tumors with poor response to radioiodine or external beam radiotherapies(3).

A pattern of genetic mutations has been identified in thyroid cancers of follicular cell derivation(5) with specific genotype-phenotype correlations, providing the opportunity to apply molecular tools to improve an area that suffers significant inter-observer variability with cytologic and histologic approaches(6). There is evidence of sequential progression of well-differentiated thyroid carcinoma through the spectrum of poorly differentiated to undifferentiated thyroid carcinoma, supported by the presence of pre- or co-existing well-differentiated thyroid carcinoma and the common core of genetic loci with identical allelic imbalances in co-existing well-differentiated components(7;8).

Genetic alterations in signaling pathways
Alterations in key signaling pathways represent the hallmark of distinct forms of thyroid neoplasia.

Hyperfunctioning thyroid nodules
Thyroid-stimulating hormone (TSH) activates the Gs/adenyl cyclase/cyclic adenosine monophosphate (cAMP) cascade on binding to the TSH receptor (TSH-R), thereby regulating thyroid hormone synthesis and growth of follicular cells. The TSHR is a classical seven–transmembrane domain-G protein–coupled receptor. Gain-of-function mutations of TSHR (14q31) and GNAS1Gs (20q13), which constitutively activate cAMP, occur in hyperfunctioning follicular adenomas (FA) that exhibit a characteristic papillary architecture but lack the nuclear features of PTC(9). In contrast, activating mutations of TSHR and GNAS1 in thyroid malignancies are rare, consistent with the clinical knowledge that hyperfunctioning thyroid nodules are highly unlikely to be malignant.

Well-differentiated thyroid carcinoma
This commonest form of thyroid cancer is associated with mutations or rearrangements that activate the MAPK pathway. Exclusive, non-overlapping activating events involving RET, NTRK, BRAF, or RAS are detectable in nearly 70% of cases(5).

The RET proto-oncogene (10q11.2), encodes a transmembrane receptor tyrosine kinase normally expressed in the central and peripheral nervous systems, enteric neurons and developing kidney. Glial-derived neurotrophic factor (GDNF) ligands and GDNF receptor- (GFR ) bind the extracellular domain of RET to activate several signaling pathways. Gain-of-function RET mutations are involved in sporadic and familial C-cell-derived medullary thyroid carcinoma(10). In contrast, chimeric oncogenes, designated RET/PTC, are implicated in the development of PTC(11). Somatic chromosomal rearrangement leads to fusion of the 3′-terminal sequence of RET encoding the tyrosine kinase domain and 5′ sequences of heterologous genes. Although wild type-RET is not normally expressed in follicular cells, RET/PTC chimeric oncproteins lacking a signal peptide and transmembrane domain are expressed in the cytoplasma of follicular cells under the control of the newly acquired promoters. Ligand-independent tyrosine phosphorylation is induced by constitutive dimerization of the fusion proteins.

The overall prevalence of RET/PTC rearrangements in PTC varies from 3% to 85%, depending on geographic location and detection methods; a reasonable range is 13-43%. More than 15 RET/PTC rearrangements have been described in sporadic and radiation-associated PTC. The most common in sporadic PTC are chromosome 10 inversions, RET/PTC1 (fusion partner CCDC6, formerly H4) and RET/PTC3 (fusion partner NcoA4, formerly ele1).
The high incidence of RET rearrangements in childhood PTCs following the Chernobyl accident suggests a role for radiation damage in their genesis. Analysis of chromatin patterns in thyroid follicular cells identified physical proximity of the partners involved in the illegitimate recombination of these rearrangements, supporting radiation-related susceptibility.

Although thyroid-targeted expression of RET/PTC1 or RET/PTC3 induces thyroid neoplasms in transgenic mice (13,14), they do not recapitulate the metastatic phenotype without additional alterations. The high frequency of RET rearrangements in subclinical papillary microcarcinomas indicates that they represent early events in the neoplastic process (15). Heterogeneity of RET rearrangements in a single tumor has been interpreted to indicate a relatively late event, however, an alternative interpretation is that multiple and distinct rearrangements signify multifocal transforming events in benign clonal neoplasms (16).

RET/PTC rearrangements have been reported in Hashimoto’s thyroiditis (17), restricting their value as a diagnostic marker, but supporting the concept of thyroid dysplasia (18). In nodules, the presence of such a rearrangement should be considered indicative of malignancy.

Mutations in BRAF were recently identified as the most common MAPK effector, identified in 29-69% of PTCs. The proto-oncogene BRAF (7q24) encodes a serine/threonine kinase that transduces signals through the RAS/RAF/MEK/ERK cascade. Gain-of-function BRAF mutation is implicated in melanoma and colon carcinoma. Several point mutations of exon 15 have been found in thyroid cancers. The most common event, BRAFΔV600E, is characteristic of classical variant PTC (19-23). Follicular variant PTCs do not usually harbor BRAF mutations but the rare BRAFΔK601E mutation has been described in these lesions (24). The in-frame VK600-1E deletion (BRAFΔV600-1E) has been detected in a solid variant PTC (25).

BRAF mutations are not found in FC, but are identified in up to 13% of poorly differentiated and 35% of undifferentiated thyroid carcinomas (22;26;27), supporting a model of progression of this disease. Some studies have reported that BRAF mutations in PTC correlate with distant metastasis and more advanced clinical stage, and occur at a significantly higher frequency in older patients; a gender difference in incidence is still controversial. There is reported to be a high prevalence of BRAF mutations in the aggressive tall cell variant (55-100%). It has been suggested, therefore, that BRAF-mutated PTCs behave more aggressively, but this remains to be proven, since BRAF mutations are also found in clinically insignificant microcarcinomas. Protein levels of BRAF show no association with mutation (28). A rare chromosomal rearrangement (AKAP-BRAF) representing yet another form of paracentric inversion has been identified in radiation-associated thyroid cancer (29).

The NTRK1 (TRK, TRKA) proto-oncogene (1q22), encoding the transmembrane tyrosine kinase receptor for nerve growth factor is also the subject of rare rearrangements in PTC. NTRK1 is typically restricted to neurons of the sensory spinal and cranial ganglia of neural crest origin. NTRK1 rearrangements show ectopic expression and constitutive activation of the tyrosine kinase analogous to RET rearrangements, have been noted in 5-13% of sporadic but in only 3% of post-Chernobyl childhood PTCs (5). To date, TPM3, TPR, and TFG, have been identified as fusion partners forming chimeric oncogenes designated as TRK, TRK-T1 and T2, TRK-T3, respectively. The prevalence of each fusion type is nearly equal in sporadic PTC while TPM3-NTRK is more prevalent in post-Chernobyl childhood PTC.

Three RAS proto-oncogenes HRAS (11p11), KRAS2 (12p12), and NRAS (1p13) are implicated in human tumorigenesis. Mutations involving codon 61 of HRAS and NRAS have been reported with variable frequency in thyroid neoplasms (5). RAS mutations are more common in iodide-deficient than iodide-sufficient areas and in lesions with follicular architecture (including FC and follicular variant PTC) than in typical PTC. RAS mutations are rare in radiation-induced thyroid cancers.

Some have argued that the presence of RAS mutations in benign tumors implicates this as an early event in thyroid tumorigenesis, however, the high degree of observer variation in the diagnosis of FA, FC and follicular-variant PTC may explain this finding. Moreover, there is considerable variability in the prevalence of RAS mutations in different series; this may be attributable to the techniques used. A low incidence in well-differentiated thyroid carcinoma was found using the most stringent techniques and highly specific analysis suggests that RAS mutations are more common in poorly differentiated and undifferentiated thyroid carcinoma, implicating this mutation in tumor progression (30).

Genetic alterations in differentiation factors

The peroxisome proliferator activated receptor gamma (PPARγ), encoded by PPARG (3p25), is a member of the steroid nuclear hormone receptor superfamily. PAX8-PPARG rearrangements were first identified in thyroid neoplasms with a cytogenetically detectable translocation t(2;3)(q13;p25) that...
Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD

generates a chimeric gene encoding the DNA binding domain of the thyroid-specific transcription factor paired-box gene (PAX8) and domains A-F of PPAR (31). The PPARG rearrangement acts through a dominant-negative effect on the transcriptional activity of wild-type PPAR. The fusion oncprotein contributes to malignant transformation by targeting several cellular pathways, at least some of which are normally engaged by PPAR.

The PPARG rearrangement appears to be almost restricted to FA (0-31%) and FC (25-63%), with the initial suggestion that it correlates with a vasculo-invasive phenotype (32). The presence of a PAX8-PPARG rearrangement in follicular variant PTC is controversial and it has not been detected in poorly differentiated and undifferentiated thyroid carcinomas. The extent to which these findings will broaden the application of PPARγ agonists to the treatment of thyroid cancer remains to be determined.

Genetic alterations in tumors with oncocytic change
Proliferation of oncocytic follicular cells gives rise to hyperplasia, adenoma, and carcinomas. Until recently, oncocytic thyroid neoplasms were considered distinct from other follicular cell-derived tumors and the criteria for malignancy were based solely on invasive behavior. However, there were reports of non-invasive oncocytic neoplasms considered to be adenomas that gave rise to lymph node metastases. The identification of RET/PTC rearrangements in oncocytic lesions was initially interpreted as indicative of a lack of diagnostic specificity of the rearrangements, but since the behavior of such lesions includes lymph node metastases, it should rather be considered a more accurate marker of metastatic potential and a diagnostic criterion of oncocytic PTC (33). Indeed, the identification of RET rearrangements and BRAF mutations in oncocytic tumors has resulted in the classification of some oncocytic neoplasms as variants of PTC (24).

Mitochondrial DNA (mtDNA) deletion and/or point mutation are frequent in non-neoplastic and neoplastic thyroid cells displaying morphologic oncocytic change, suggesting a contribution of mtDNA alterations to the oncocytic process (34-35). However, it is not clear if this represents a separate and distinct process from neoplastic transformation. Several studies have demonstrated a positive impact of mtDNA mutation on cell growth and tumorigenicity. The identification of somatic and germline missense mutations in GRIM-19 (19p13.2) gene in oncocytic FC and PTC but not in oncocytic FA and non-oncocytic thyroid carcinoma is consistent with a dual function of this gene in mitochondrial metabolism and cell death (36).

Growth factors in thyroid cancer
The genetic events identified in thyroid cancer indicate a pattern of cumulative alterations that play a role in initiation, progression and dedifferentiation. However, there is a spectrum of biologic behaviors of well-differentiated thyroid cancer; many are microscopic lesions identified incidentally, others are slowly growing but non-metastatic neoplasms, and some give rise to local and distant metastases. Signaling via growth factors and their receptors is considered essential for cancer progression and some of these have been identified as modifiers of transformed thyroid cell behavior.

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) are important regulators of angiogenesis and tumorigenesis. Expression of FGF2 (basic FGF) is increased in thyroid cancer. Contrasting expression patterns of FGFRs are identified in non-neoplastic and neoplastic thyroid. Normal adult thyroid expresses FGFR2; in contrast, FGFRs1, 3 and 4 are expressed in thyroid carcinoma (37). FGFR4 expression appears to be restricted to the aggressive phenotype with conspicuous absence in more indolent forms of thyroid carcinoma. Thus far, however, no mutations or rearrangements involving members of the FGFR family have been identified in thyroid cancer. Interestingly, the FGFR2-IIIb isoform, which is down-regulated in neoplastic cells by CpG methylation, can be upregulated by the demethylating agent 5’-Aza-dc that restores FGFR2 protein expression in those cells (38). This re-expression of FGFR2-IIIb competes with FGFR1 for the adaptor immediate substrate FRS2 to impede signaling upstream of the RAS/BRAF/MAPK pathway, thereby overcoming the effects of intrinsic mutations, and also inhibits invasiveness of thyroid cancer cells (38).

The receptor tyrosine kinase MET (7q31) is the main signaling receptor for hepatocyte growth factor (HGF). Over-expression of MET is frequent in PTC and rare in other histological types of thyroid tumors (39-40). HGF–MET signaling has been implicated in PTC cell motility and invasiveness while promoting angiogenesis. Point mutations involving MET have been found in 7% of well-differentiated thyroid carcinomas as either somatic or germline events; no association was observed between MET mutation and protein level (41). MET over-expression in thyroid carcinoma is thought to be regulated by transcriptional or posttranscriptional mechanisms as a secondary effect. For example, mutant RAS or
**Molecular Tests for Thyroid Cancer**

Sylvia L. Asa, MD, PhD

\textit{RET} can induce MET over-expression in thyroid follicular cells. Therefore, the pathogenetic significance of MET expression in PTC remains to be verified.

The epidermal growth factor receptor (EGFR) family includes EGFR (erbB1, HER1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). Thyroid tumors over-express EGFRs and ligands but no activating mutations or DNA amplification of EGFRs have been found in human thyroid neoplasia(5). Increased expression of HER1 correlates with poor prognosis, whereas HER2 has no clear prognostic significance(42).

Overexpression of vascular endothelial growth factor (VEGF) ligands (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) has been reported in thyroid carcinomas. VEGF-A and -B are angiogenic; VEGF-C and -D are primarily lymphangiogenic. Over-expression of VEGF-C and -D in PTC correlates with density of lymphatics and lymph node metastasis(5). Enhanced expression of VEGF-A may contribute to the papillary morphogenesis of PTC.

**Cell cycle regulation**

The growth activity of well-differentiated thyroid carcinoma is low compared to poorly differentiated and undifferentiated thyroid carcinomas. Altered expression of cell-cycle regulators likely governs these differences of growth activity. Although nuclear immunoreactivity for cyclin D1 (CCND1, 11q13) and cyclin E (CCNE1, 19q12) are not detectable in normal thyroid follicular cells, expression of cyclin D1 and E is observed in approximately 30% and 76% of PTCs respectively(43). Furthermore, cyclin D1 over-expression correlates with metastatic spread in PTC and significant over-expression of cyclin D1 is observed in undifferentiated thyroid carcinoma(44;45). No amplification or major genetic alterations of CCND1 and CCNE1, indicating that over-expression of cyclins is due to secondary or epigenetic changes.

CDK inhibitors are commonly downregulated in thyroid neoplasia. There is progressive loss of p21CIP1 (CDKN1A, 6p21) with advancing tumor stage of PTC, and 13% of PTCs harbor CDKN1A deletions(5). Normal and hyperplastic follicular cells show strong immunoreactivity for p27KIP1 (CDKN1A, 12p13), whereas p27KIP1 expression is significantly reduced in metastatic PTC and undifferentiated thyroid carcinoma(5;45;46). Point mutations of CDKN2A (9p21), encoding p16INK4A, are rare in thyroid tumors. However, LOH in the region of CDKN2A is associated with FC and undifferentiated thyroid carcinoma (50%). Moreover, methylation of the 5’ CpG island of CDKN2A is detected in 30% of thyroid neoplasms(5).

The tumor suppressor gene TP53 (17p53) appears to be a critical gatekeeper of progression from indolent to lethal thyroid cancers, evidenced by the restricted mutation of TP53 in aggressive lesions (17-38% of poorly differentiated thyroid carcinoma and 67-88% of undifferentiated thyroid carcinoma vs 0-9% of well differentiated thyroid carcinoma)(5). Activation of wild type p53 can lead to G1 cell cycle arrest via p21CIP1 and apoptotic cell death, preventing replication of cells with damaged DNA. Conversely, loss of function mutation of TP53 induces genomic instability due to weakened DNA repair systems and subsequent cancer progression.

**Adhesion molecules and the extracellular matrix**

Cadherins are single-transmembrane calcium-dependent cell-cell adhesion proteins. There are three classical cadherins: neuronal (N)-, placental (P)- and epithelial (E)-cadherin. E-cadherin (CDH1, 16q22) is highly expressed in normal thyroid, FAs, and minimally invasive FCs, however, in widely invasive, recurrent or metastatic thyroid carcinomas it is low or absent(47). Its expression in undifferentiated thyroid carcinoma is extremely low. Somatic mutations of CDH1 are infrequent, and this change is more likely attributable to methylation of the CDH1 promoter. Cadherin switching, documented in several cancers, is evidenced by neo-expression of P-cadherin in areas of squamous metaplasia of PTC. N-cadherin expression, observed in several human thyroid carcinoma cell lines that lack E-cadherin, is not found in vivo(5).

\textit{-catenin} (CTNNB1, 3p22-21.3), a cadherin-mediated adhesion regulator and mediator of Wnt/ \textit{-catenin signaling that targets cyclin D1 and c-myc, is expressed as a membranous protein in normal thyroid follicular cells. It is progressively reduced with tumor dedifferentiation. Nuclear localization, induced by CTNNB1 mutation, is restricted to poorly differentiated (up to 25%) and undifferentiated thyroid carcinoma (up to 66%)(48;49), implicating CTNNB1 mutation in progression to poorly differentiated or undifferentiated thyroid carcinomas.

Somatic/germline mutations in the APC/\textit{-catenin pathway were thought to be the molecular basis of the peculiar cribriform morular variant of PTC that is usually associated with familial
adenomatous polyposis(3). Some investigators have questioned the role of APC/\-catenin in these lesions that also harbor RET/PTC gene rearrangements(50).

**Fibronectin** encoded by FN1 (2q35) regulates cell adhesion, migration, tumor invasion and metastasis. Fibronectin expression is upregulated in well-differentiated thyroid carcinoma compared with normal thyroid tissue(40;51). In contrast, reduced fibronectin expression is documented in transformed cell lines and at the periphery of invasive well-differentiated thyroid carcinoma(52). Consistent with the presence of a vitamin D response element in the FN1 promoter, vitamin D compounds increase fibronectin expression and restore thyroid cancer cell adhesiveness, thereby representing a therapeutic target for metastatic thyroid cancers(53).

**CEACAM1**, a member of the CEA immunoglobulin superfamily, is a putative tumor suppressor based on diminished expression in some solid neoplasms such as colorectal carcinoma and a putative oncogene based on its over-expression in tumors such as non-small cell lung cancer. CEACAM1 is expressed in thyroid carcinoma cell lines derived from tumors that exhibit aggressive behavior. Overexpression reduces cell cycle progression but enhances cell adhesion and promotes tumor invasiveness. CEACAM1 is not appreciably expressed in normal thyroid tissue or benign thyroid tumors. CEACAM1 reactivity is associated with metastatic spread but not with increased tumor size. These findings identify CEACAM1 as a unique mediator that restricts tumor growth while increasing metastatic potential(54).

These findings indicate that loss of adhesion serves to promote tumor invasiveness and enhance metastatic potential in thyroid cancer. The frequently indolent nature of thyroid microcarcinomas harboring genetic defects in initiation factors (RET/PTC-RAS-BRAF) but lacking defects in adhesive functions emphasizes the importance of progression factors including cell adhesion in dictating tumor behavior.

**Genomic instability**

Chromosomal instability has been identified in FAs and FCs that are frequently aneuploid with a high prevalence of loss of heterozygosity (LOH) involving multiple chromosomal regions. This contrasts with the diploid or near-diploid content of PTC that rarely exhibits LOH, supporting the hypothesis that these lesions develop via discrete molecular pathways(5). Microsatellite instability occurs in benign and malignant thyroid lesions.

Genomic instability may be important in thyroid cancer progression. Mutant HRASV12 or BRAFV600E induces genomic instability(5), suggesting that oncogenic activation of the MAPK signaling pathway may be responsible for additional somatic mutations in transformed cells.

**Biomarkers of thyroid cancer**

The data summarized above provide novel diagnostic and/or prognostic tools for patient management. Genetic defects such as RET rearrangements and BRAF mutations can now be detected preoperatively in cytologic specimens to improve diagnosis and determine appropriate therapy(55;56). Under-expression of p27, over-expression of cyclin D1, loss of fibronectin or E-cadherin and upregulation of CEACAM1 are predictors of lymph node metastases in PTC. -catenin nuclear localization and p53 stabilization are characteristics of poorly differentiated and undifferentiated carcinoma respectively.

Controversies in patient management have also been addressed by the analysis of molecular changes that underlie tumorigenesis. As an example, multifocality is a unique and frequently encountered characteristic of PTC(15;57). The presence of multiple microcarcinomas in the same gland has been the rationale for aggressive surgery in patients with low-risk PTC, since it was considered to indicate intrathyroidal dissemination via lymphatics from a primary tumor. However, clonality assessment using RET/PTC rearrangement or X-chromosome inactivation analyses has revealed distinct genetic profiles among these foci in a given gland in more than two thirds of cases, supporting a coincidental and unrelated origin of the multiple nodules. This interpretation of multifocal papillary microcarcinoma defines an entity with considerably less aggressive implications compared with the alternative diagnosis of metastatic thyroid carcinoma. Similarly, the finding that PTCs with BRAF mutations are less often multicentric than those with wild-type BRAF also suggests potentially less aggressive behavior of multiple microcarcinomas(58).

Gene profiling of PTC by cDNA microarrays have identified up-regulated genes, over-expression of MET, FN1, LGALS3 (galectin-3), KRT19 (cytokeratin 19), CITED1 (cpb/p300-interacting transactivator-1), and PDGFA (platelet-derived growth factor ) and these have been confirmed at the protein level by immunohistochemistry(40;59). Some of these genes may play a role in determining morphological and biological characteristics of PTC, and some may be involved in the carcinogenic process. Indeed,
Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD

malignant transformation has been described after transfection of LGALS3 (galectin-3) into thyroid follicular cells. Consistent with microarray analysis, immunohistochemical detection for galectin-3, fibronectin, and cytokeratin-19 has been proposed as an ancillary tool for the diagnosis of thyroid cancer in cytologic and pathologic specimens(60). Immunopositivity of HBME-1 using an antibody raised against mesothelial cells is seen in more than half of malignancies of thyroid follicular cell derivation in pathologic thyroid specimens(61), however, its corresponding antigen, proteins, or gene remain to be elucidated.

Distinctive gene expression profiles are also observed between PTC and FC. PTC shows over-expression of CITED1, CLDN10 (claudin-10), and IGFBP6 (insulin-like growth factor), while FC shows no expression of CLDN10 and under-expression of IGFBP6 and/or CAV1 (caveolin-1) and CAV2 (caveolin-2)(62). Such gene panels require wider validation to permit adoption as ancillary diagnostic and prognostic biomarkers for thyroid cancer.

Future directions
Thyroid neoplasms show a wide range of biological behaviors from indolence to highly aggressive, invasive, and metastatic cancers. The identification in the majority of sporadic PTCs of exclusive non-overlapping activating mutations or rearrangements along the MAPK pathway including RET/PTC, NTRK1, RAS and BRAF has greatly aided our understanding of thyroid cell transformation. The challenge now will be to identify modifying factors, particularly those mediating tissue, vascular, and lymphatic invasion, that represent features of virulent forms of the human disease. These studies will offer insights into the stepwise progression of neoplasia, providing unique signatures for accurate diagnosis and prognosis. The molecular alterations identified in this disease also represent targets for early clinical trials aiming at tailored multimodal approaches to the treatment of thyroid cancers.

References
3. DeLellis RA, Lloyd RV, Heitz PU, Eng C. Pathology and Genetics of Tumours of Endocrine Organs. WHO Classification of Tumours. Lyons, France, IARC Press. 2004
Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD


27. Soares P, Trovisco V, Rocha AS et al. BRAF mutations typical of papillary thyroid carcinoma are more frequent detected in undifferentiated than in insular and insular-like poorly differentiated carcinomas. Virchows Arch 2004.


Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD


Molecular Tests for Thyroid Cancer
Sylvia L. Asa, MD, PhD


DNA Fingerprinting

Jennifer L. Hunt, M.D., M.Ed.
Section Head, Surgical Pathology
Director, Head and Neck and Endocrine Pathology
Director, Molecular Anatomic Pathology Unit

Agenda

• Case presentation
• Molecular identity testing
• Risk management

Case Presentation

• 64 year old man with prostate biopsy
  — Carcinoma identified

Case Presentation

• Prostatectomy
  — Benign
Case Presentation

Question:
Did the sample with cancer come from this patient?

Molecular Approaches to Identification of Tissue Contamination in Surgical Pathology Sections

A Microdissection and Molecular Genotyping Assay to Confirm the Identity of Tissue Floaters in Paraffin-Embedded Tissue Blocks

DNA Polymorphisms

- Define identity
  - Association with phenotype

- Define risk for disease
  - APO-E genotype
  - Alzheimer’s disease
  - Cardiovascular disease

- Complex relationships

Molecular DNA Identity Testing

- Polymorphisms
- Testing
- Other applications

DNA Polymorphism Inheritance

- Polymorphisms are inherited
- 1 every 1000 basepairs
- Different types
Types of Polymorphism

• Single nucleotide polymorphisms (SNPs)

• Short tandem repeats (STRs)
  – Microsatellites

• Variable nucleotide tandem repeats (VNTRs)

DNA Polymorphisms

• Short tandem repeats
  – 2 to 7 basepairs in length
  – Dinucleotide, Trinucleotide, Tetranucleotide...
  – Repeated a variable number of times

\[
\text{ATCG ATCG ATCG ATCG ATCG ATCG}
\]

DNA Polymorphisms

Allele 1

\[
\text{ATCG ATCG ATCG ATCG}
\]

Allele 2

\[
\text{ATCG ATCG}
\]

DNA Polymorphisms

• Informative

• Heterozygous

DNA Polymorphisms

• Non-informative

• Homozygous

PCR Short Tandem Repeats

Allele 1

\[
\text{ATCG ATCG ATCG ATCG}
\]

 Allele 2

\[
\text{ATCG ATCG}
\]
**PCR Short Tandem Repeats**

- **Larger**
- **Smaller**

**Amount of PCR Product**

- **More**
- **Less**

**Lab DNA Fingerprinting**

- Microdissect specimen
  - Floater, whole sections, biopsies, FNA cells
- Extract DNA
- Perform PCR
- Analyze PCR amplicon
- Compare samples

**Microdissection**

- By hand
  - Microscope
  - Implement
- Laser capture

**Microdissection**

- Implement
Identity testing

- Also known as...
  - DNA Profile
  - Genotype
  - DNA Fingerprint

DNA Fingerprinting Kits

- Multiplex assays
  - Multiple primers all in one tube
  - Assess short tandem repeats
  - Assess (X, Y) status

- Capillary electrophoresis analysis
Multiplex PCR Product Analysis

- 5 Different Dyes
- 16 Genetic Loci

Comparing Two Samples

Other Applications

- Floaters and contaminants
- Gestational trophoblastic disease
- Transplant
  - Tumors
  - Chimerism
- Paternity testing
- Forensic testing

Contaminants and Floaters

- Contaminants present: 0.6 to 3% of slides
- Malignant contaminants: 6 to 13% of slides

Contaminants and Floaters

- Contaminant on slide only: 59 to 73%
- Contaminant in block: 16 to 28%

Contaminants and Floaters

- Severe diagnostic difficulty: 0.1 to 0.4% of slides
  - Molecular resolution: 21 cases (100%) / 16 (76%) discordant
Impact

• Floaters & contaminants are common
• Many are malignant
• Some create diagnostic difficulties
• Can be resolved using molecular

When To Test

• Floaters
  — Malignant or change in diagnosis
• Question of specimen mix-up
• Benign resection & malignant biopsy

Guidelines

• Don’t cut into blocks
  — Tissue needed!
• Talk to your clinicians
• Talk to risk management or legal
• Look around the lab
  — Potential sources
  — Unusual diagnoses
DNA Fingerprinting and Identity Testing

DNA Fingerprinting

In the past, immunohistochemical stains were used with some variable success, to assess for the ABO blood group antigens or HLA antigens on tissue fragments that were suspected of being floaters [1, 2]. However, because biopsies can be small and can exhibit edge artifact, the staining patterns can be difficult to interpret. Recently molecular techniques have been detailed that can reliably differentiate tissues from different patients based upon the DNA fingerprinting [3-7].

In the DNA fingerprinting test, two samples are compared: a sample that is known to be from the patient (either a blood sample, or another confirmed tissue sample) and the specimen of. If the sample of interest is a floater or a carry-over contaminant, the fragments are microdissected from the glass slides. If the tissue is all suspected as being a mixed-up sample, less exact microdissection is needed. DNA is extracted from the tissue fragments and PCR is performed for a set of polymorphic markers.

The polymorphisms that are tested for in typical identify testing assays are called short tandem repeats. These are short sequences of DNA, ranging between 1 and 5 basepairs in length that are repeated for a variable number of times. The number of the repeats is what is polymorphic, since most people will harbor two alleles with different numbers of repeats. These are inherited, and thus the alleles can be traced back to the father’s and mother’s alleles. In the identity test, we usually use tetranucleotide repeats (4 basepair sequence that is repeated), but there are also mononucleotide, dinucleotide, trinucleotide, and even pentanucleotide repeats.

Most laboratories use kits that are pre-formulated to contain a large set of known robust polymorphic markers [8]. In these kits, multiple PCR primers are multiplexed so that all of the primers are in one tube and can be run with one PCR reaction. This type of complex multiplexing is difficult and requires substantial quality control and validation. However, the interpretation of the resulting genotype, or fingerprint, is usually fairly straightforward. Because the markers are highly polymorph in the general population, it is highly likely that each unique patient will have a different DNA profile at these markers. If the genotypes match at multiple loci, it is statistically highly likely that they came from the same patient. If they do not match, however, the specimens definitely did not come from the same patient.

This type of assay can also be used to identify specimen floaters or carryover artifacts in paraffin blocks. In cases of identity testing in paraffin blocks, it is extremely useful to be able to test another sample from the patient that is known to contain genomic DNA (such as blood, or another biopsy sampling from another time) and to also test the tissue from which the putative floater is suspected of being derived. In busy anatomic pathology practices, it may be difficult to determine which case a floater arose from. But, when possible, this adds an additional confirmatory layer to molecular testing for carryover artifact or floater contamination.
Many different types of samples are amenable to this type of molecular testing. It can be performed on recut unstained slides, which are the most successful source of tissue [9]. Even single fragments on stained H&E slides, however, yield fairly good results with this assay. The smaller the fragment, the more likely it is to be difficult to amplify.

DNA fingerprinting is an extremely robust and powerful molecular technique. These assays have gained public awareness as they are commonly used in forensic pathology, for identification of remains and for linking suspects with crimes based upon the presence of body fluids or residual cells. Although the panel of polymorphic markers that are used in forensic testing varies from that used clinically [10], the techniques and interpretation are essentially identical.

The technique of DNA fingerprinting also has another very valuable application in the arena of transplant pathology. In bone marrow transplantation, the percentage of donor and recipient cells, either in peripheral blood or in the bone marrow sampling, can be ascertained in a semi-quantitative manner, using capillary electrophoresis [11]. When the recipient and the donor are related, as is often the case, multiple polymorphic markers may have to be tested in order to find informative alleles, as related individuals will be expected to share genotypes at more genetic loci than those who are not genetically related.

The DNA fingerprinting assay can also be useful in very rare cases in which a transplant patient develops a carcinoma that is suspected of being of donor origin [12, 13]. Genotyping of the tumor can prove that it originated from the recipient or the donor cells. The test can also be used to identify a complete molar pregnancy, based on the presence of paternal-only alleles [14, 15].
References