Molecular Pathology and the Molecular Diagnostics Lab

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Kathy Gabrielson’s Course 9-28-11
Slides: CG, KM and JRE
Introduction

- What is molecular pathology?
- What’s it used for?
- Who’s doing it?
- How?
Molecular Pathology

- Use of nucleic acid-based tests to determine a diagnosis or prognosis
  - Includes hybridization, PCR, ISH, blotting and sequencing
  - Generally doesn’t include protein assays or antibody detection (however some define it more broadly). The field typically includes both molecules testing in tubes and slides (cytogenetics).
What is molecular pathology?

- Four main areas:
  - Infectious diseases
  - Identity testing (HLA, forensic)
  - Cancer
  - Classical genetics
What’s it used for in cancer?

- Cancer diagnosis
- Cancer prognosis
- Minimal residual disease detection
- Transplant monitoring
- Chemosensitivity Prediction (infancy)!
Who’s doing it?

- Labs
- People
- Qualifications
Labs performing Molecular Testing

According to GeneTests, there are:

- 604 laboratories testing for
- 1,435 diseases (clinical)
- 281 diseases (research)

ref: (www.genetests.org)!!
Approximately 120 labs participate in the CAP Check Sample Program in Molecular Oncology (started 1992)

- Most perform common Ig and TCR analysis for leukemia/lymphoma
- Other tests include translocation testing and bone marrow engraftment
People

- Association for Molecular Pathology has ~1,100 members
- CLIA ‘88 and JCAHO regulations!
  - Imposes educational and training requirements
  - Need to develop QA and QC programs
  - Clinical validation
  - Lab inspections
Qualifications

- **Lab director:**
  - MD or DO with 2 years pathology training or experience
  - PhD with board certification or 4 years experience
- **Technical supervisor:**
  - MD, DO, PhD with 1 year experience
  - MA with 2 years experience
  - BA with 4 years experience
Lab environment

Recommendations:

- Three rooms, 2 clean + 1 “dirty” (hallway inbetween)
- Unidirectional flow of specimens
- Dedicated equipment
- Air handling
- Dedicated lab coats and frequent glove changes
What does a lab cost?

Recent estimate: ~$180,000 capital

Clinical Diagnosis & Management by Laboratory Methods, 19th ed.
CLIA ’88
Clinical Validation

- FDA approved test, lab must verify accuracy, precision, reportable range
Clinical Validation

- Home-brew tests, lab must verify:
  - Accuracy (min 20 samples)
  - Precision
  - Reportable range
  - Analytical sensitivity and specificity
  - Reference intervals
  - Standard Operating Procedure (SOP), basically a detailed protocol
Technology

- Technology is key and can be highly limiting
- Common Tools (expensive)
  - Cell sorter (1)
  - DNA robot (2)
  - Spectrophotometer (1)
  - Speedvac (1)
  - PCR setup hoods (6)
  - Thermocyclers (10)
  - Real time PCR thermocyclers (3)
  - Capillary electrophoresis (DNA sequencers) (3)
  - Flow cytometer (1)
Methods

- Cell separation or microdissection
- RNA and DNA isolation
- PCR, +/- restriction digest, CE
- PCR and CE
- PCR, restriction digest, gel (1 assay still)
- Q-PCR and RT-Q-PCR
- PCR, bead attachment, flow cytometry
- DNA sequencing
Mutations and germline variants

- Cleanliness of the sample
  - Many clinical samples are not homogeneous
  - Microdissection, cell sorting

- Easy to detect vs. hard to detect?
  - Translocations
  - Base substitutions
  - Loss of heterozygosity
Capillary Electrophoresis (CE)

- Size and color of DNA molecules
- 30-800 bases, up to 5 colors
- Can be used for sizing or sequencing

ABI 310
(monocapillary)
Realtime PCR vs. gel

- Loose the molecular weight determination (melt curve is somewhat of a surrogate though)
- Gain specificity through use of probes (sybr green only probably inappropriate for clinical work)
- Gain quantification (linear over >8 orders of magnitude)
- Removes the next analysis step (e.g. no CE, etc)
- Closed tube system, so safer (no amplicon floating around).
Research vs. Clinical

- **Labeling**, labeling, labeling
- **Cross contamination cannot** occur
- **How will the test result be used?**
  - Need to validate assays, even if a “kit” from a company
    - Accuracy, sensitivity, specificity, positive predictive value, negative predictive value
  - **Need to be right**
    - 100% vs. >95%
- **Probability of experiment working**
  - >30% vs. >98%
  - Careful and detailed SOPs, extensive tech training
Infectious Diseases

- Two examples: HIV and HPV
HIV testing

- The 800 pound guerilla in Molecular Pathology
- Comprises 80-90% of UM workload (~12000 cases/yr)
HIV genotyping

- Goal in HIV therapy is viral suppression, not eradication (currently)
- Viral mutations confer resistance to specific drugs, depending on their mechanism of action. Can be discovered by genotyping (DNA sequencing)
- Therapy can be adjusted to avoid ineffective anti-retrovirals
HIV testing: genotyping for drug resistance mutations

ABI 3130
16 caps

1 m

The 3130 Series Systems include the powerful 3130xl Genetic Analyzer—a robust, fully automated 16-capillary system, and the 3130 Genetic Analyzer, which offers identical capabilities in an economical, upgradable 4-capillary configuration.
Infectious disease testing: human papilloma virus (HPV)

- HPV causes almost all cervical carcinomas
- Two categories of HPV: high risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and low risk (types 6, 11, 42, 43, 44)
- May be especially critical in resource poor environments where there are no pap smears and the current vaccines are too expensive
Infectious disease testing: Improving the Pap smear

- Relative risk of developing high grade dysplasia (premalignancy) if infected with high risk HPV = 76-fold
Infectious disease testing: Improving the Pap smear

- End result: cervical carcinoma kills 4,100 US women/yr
Infectious disease testing: Improving the Pap smear

- Hybrid capture HPV detection

1. Lyse cervical cells
2. Hybridize with cRNA
3. Bind with anti-DNA/RNA antibodies
4. Add labeled anti-DNA/RNA antibodies
5. Detect label
Infectious disease testing: Improving the Pap smear

- Sensitivity for identifying dysplasia when cytology is abnormal:
  - Repeat Pap smear 67-85%
  - Hybrid capture DNA test 82-100%

- Specificity of DNA test ~64%
What’s it used for in cancer?

- Cancer predisposition
- Cancer diagnosis
- Cancer prognosis
- Minimal residual disease detection
- Transplant monitoring
- Chemosensitivity Prediction (infancy)!
Cancer Predisposition-Examples

- I1307K, mutation in the APC gene. Prelavent in patients of Ashkenazi Jewish decent. Predisposes to CRC
- MMR gene germline defects. The cause of Lynch syndrome (CRC, etc)
- BRCA2 and Palb2 (will be in Science) predisposes to Breast, Ovarian, Pancreatic cancers
- Why do we need to know about these mutations?
What’s it used for in cancer?

- Cancer predisposition
- **Cancer diagnosis**
- Cancer prognosis
- Minimal residual disease detection
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- Chemosensitivity Prediction (infancy)!
Diagnosis--Chronic Myelogenous leukemia

- Cytogenetics!
  - CML was first genetic malignancy known
    (Philadelphia chromosome) translocation of chr 22
    (BCR) to chr 9 (ABL)
  - Bcr-Abl translocations can be detected in cells using
    FISH (limit of detection about 1%)
  - Can also be tested by PCR (LOD ~1x10^{-5})
t(9:22), Bcr-Abl, Philadelphia Chromosome
BCR-ABL qRT-PCR

- RNA
- Real Time PCR- quantitative
- Control gene = ABL
- Controls and Standards
- Samples are batched to eliminate run to run variability
Chronic leukemia—treatment

- Gleevec (imatinib) was first small molecule designer drug
- Wonder drug—places most CML patients into remission, maintenance
- Rituxan (monoclonal antibody against lymphocyte surface antigen) also great drug for CLL
Gleevec/Imatinib mesylate/STI571 (Novartis)

- Small molecule tyrosine kinase inhibitor with activity against PDGF-R, c-kit, and bcr/abl.
- Highly active in inducing CHR in CP CML patients (>90%).
- Disease progression reported even for those achieving molecular CRs.
- Multiple mechanisms of resistance to imatinib mesylate in relapsed patients.
CML treatment—effective?
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Prognosis--FLT3 Activating mutations occur in ~30% of AML cases

- FLT3 Internal Tandem Duplication (ITD) (~25%):
  - 3 to > 400 bp insertions into the juxtamembrane region, always in-frame.

- FLT3 kinase domain mutations (~7%):
  - Most commonly at D835

- Both mutations constitutively activate the tyrosine kinase function of the receptor.

- For a molecular marker to be used, it must have a significant effect on prognosis (e.g. a 10% difference is unlikely to ever be used by clinicians)
Flt3 ITD mutation and Prognosis

AML patients harboring a FLT3 mutation have a much worse outcome...

Frohling et al, 2002
Flt3 mutation detection

Results of Flt3 multiplex PRC with CE detection
What’s it used for in cancer?

- Cancer predisposition
- Cancer diagnosis
- Cancer prognosis
- Minimal residual disease detection (including molecular relapse)
- Transplant monitoring
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Molecular Relapse--Chronic leukemia

- In CML, following amount of BCR/ABL as disease marker predicts survival:
  - Decreasing or stable BCR/ABL after achieving cytogenetic remission is good
  - Increasing (after 6 months) is bad, indicates relapse
BCR/ABL molecular monitoring
MRD detection is clinically significant in CML

What’s it used for in cancer?

- Cancer predisposition
- Cancer diagnosis
- Cancer prognosis
- Minimal residual disease detection (including molecular relapse)
- Transplant monitoring
- Chemosensitivity Prediction (infancy)!
• 9-16 STRs and 1 sex-determining loci can be interrogated in one PCR reaction.
• Fluorescent PCR products are analyzed by Capillary Gel Electrophoresis (CGE)
CE Analysis of Microsatellites

- Single base sizing
- Analysis of 20 PCR products
BMT Pre-transplant Comprehensive Analysis

Patient (pre-transplant)

Donor
BMT Followup Analysis

\[
p_{2761}/(p_{2761} + d_{2319}) = 54\% \ p \quad \text{and} \quad P_{2567}/(P_{2567} + d_{2301}) = 53\% \ p
\]
What’s it used for in cancer?

- Cancer predisposition
- Cancer diagnosis
- Cancer prognosis
- Minimal residual disease detection (including molecular relapse)
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- Chemosensitivity Prediction (relative infancy)!
EGFR Inhibitors

- EGFR inhibitor FDA cleared for use in CRC (e.g. Cetuximab). EGFR expressing metastatic CRC resistant to irinotecan.

- Sensitivity correlated to the presence of EGFR amplification, and lack of Kras or Braf mutations (activated Kras acts distal to EGFR).

- EGFR by FISH for amplification. Use requires documenting presence of EGFR by IHC, although this poorly correlates with therapeutic effectiveness.

- EGFR mutation is uncommon in CRC (~1%), whereas Kras mutation is common (~30%)
Kras Mutation

- First shown by a French group to confer resistance to EGFR inhibitors (somewhat as expected).
- Detection by: DNA sequencing, high-resolution melt curve analysis, etc.
- Hopkins: manual microdissection, Kras PCR and detection of mutant vs. wildtype by melt curves of FRET probes.

Lievre, Cancer Research 66:3992, 2006
The future for molecular pathology?

- Gene chip screening for hundreds of inherited diseases
- Customized medicines based on side effect profiles
- Cancer-specific therapy identified by molecular targets in cancer cells
- Preventative actions taken based on risk profiles? whole genome sequencing at birth?