Molecular Diagnosis

Nucleic acid based testing in Oncology

Objectives

- Describe uses of NAT in
  - Oncology
    - Diagnosis, Prediction, monitoring.
  - Genetics
    - Screening, presymptomatic testing, diagnostic testing, family studies.
- Identify correct test approaches
- Evaluate clinical significance of tests
- Evaluate impact of genetic test results on individuals/families (ethical considerations)

Gain/Loss of Nucleic Acid

- Diagnosis of specific entities:
  - 1p/19q in oligodendroglioma; CLL, trisomy 12; 5q- refractory anemia; 3p- in renal clear cell carcinoma
- Prognostic/Predictive changes
  - MYCN amplification in neuroblastoma
  - HER-2/neu amplification in Breast CA
- Screening/monitoring for neoplasms
  - “Urovysion”.

Nucleic Acid Testing - Oncology

- Gross alterations in DNA content of tumors (ploidy)
- Gain/Loss of nucleic acids
- Markers of Clonality
- Oncogene/Tumor Suppressor gene mutations
- Tumor specific Translocations
- mRNA “molecular staging”
- Gene expression profiling

Molecular Diagnosis

“Nucleic Acid Testing”

- Use of specific sequence information in nucleic acids - DNA and RNA - for clinical management

MYCN and Neuroblastoma

<table>
<thead>
<tr>
<th>Feature</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYCN</td>
<td>Normal</td>
<td>Normal</td>
<td>Amplified</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td>Hyperdiploid</td>
<td>Near diploid</td>
<td>Near diploid</td>
</tr>
<tr>
<td></td>
<td>or hypotetraploid</td>
<td>or tetraploid</td>
<td>or tetraploid</td>
</tr>
<tr>
<td>1p LOH</td>
<td>&lt;5%</td>
<td>25-50%</td>
<td>80-90%</td>
</tr>
<tr>
<td>14q LOH</td>
<td>&lt;5%</td>
<td>25-50%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>7pR-K ras exp.</td>
<td>High</td>
<td>Low</td>
<td>Low or absent</td>
</tr>
<tr>
<td>11pR-C ras exp.</td>
<td>Low or truncated</td>
<td>Low or absent</td>
<td>High (full length)</td>
</tr>
<tr>
<td>7pR-C ras exp.</td>
<td>High</td>
<td>Low or absent</td>
<td>Low or absent</td>
</tr>
<tr>
<td>Age</td>
<td>Usu. &lt; 1 year</td>
<td>Usu. &gt; 1 year</td>
<td>Usu. 1–5 years</td>
</tr>
<tr>
<td>Stage</td>
<td>Usu. 1, 2, 4S</td>
<td>Usu. 3, 4</td>
<td>Usu. 3, 4</td>
</tr>
<tr>
<td>3-year survival</td>
<td>90%</td>
<td>20–50%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

Exp., expression; Usu., usually.
Chromosome 1p, 19q and Gliomas

- 1p & 19q deletions define Oligodendroglioma.
- Detected either by: FISH or PCR –based Techniques
- PCR amplification of microsatellites – normal vs tumor tissue.
- FISH: green = centromere red = 1p.

Molecular Markers of Clonality

- Antigen receptor gene rearrangements.
  - Southern Blotting: IgH, TCR; EBV termini.
  - PCR: Ig and TCR gene rearrangement.
- X-inactivation.
  - Human androgen receptor assay.
- Microsatellite allelotyping.
  - Altered microsatellites in lesional tissue vs germline (PCR amplification)
- Clonal Viral Integration
  - EBV termini, by Southern Blotting

Southern Blotting for Clonality

Immunoglobulin heavy chain locus - restriction enzyme digestion sites

Clonal Viral Integration

- EBV termini, by Southern Blotting

Antigen Receptor Gene Rearrangement

- Somatic Ig & TCR gene rearrangement:
  - V-D-J recombination
  - B- & T-cell repertoire diversity
  - Combinatorial Diversity
    - Use of different V-D- and J segments
  - Junctional Diversity
    - Addition or removal of nucleotides between D-J, V-D, or V-J segments.
    - Greatest contribution.

Clonality Testing

- Each lymphoma arises from a single cell w/ a unique Antigen receptor gene rearrangement.
- Inflammatory infiltrates
  - Lymphocytes w/ different rearrangements.
- Tests for clonality
  - Southern Blotting
  - PCR-based tests

Antigen Receptor Gene Rearrangement

- Unique V-D-J rearrangements for each event.

Southern Blotting

Immunoglobulin heavy chain locus - restriction enzyme digestion sites
Southern Blotting for Clonality

- Test based on combinatorial diversity
- Digest high DNA w/ restriction enzyme
- Separate resulting fragments by size
  - Electrophoresis (agarose gel)
- Transfer to membrane, immobilize, denature, hybridize with a probe.

PCR-based Tests for Clonality

- Primers w/ c bind to relatively invariant sites flanking sites of recombination
  - No amplification w/o recombination (primer binding sites too far)
  - Polyclonal lymphoid population
    - Mixture of PCR products (combinatorial and junctional diversity)
  - Clonal lymphoid population
    - Single Predominant PCR product
    - Differentiate by
      - Plain Electrophoresis
      - Capillary Electrophoresis
      - "heteroduplex analysis"
      - Denaturation – either on gel, or w/ "real-time" PCR

Southern Blotting vs PCR-based Tests

- PCR
  - Can use partially degraded DNA
  - 0.1-1 μg DNA
  - Quick
  - Simple
  - May go down to 1%
  - Up to 30% of rearrangements not detected due to primer binding.
  - Junctional diversity may be used to follow clone.

Clonal EBV-Integration

- EBV-associated neoplasms:
  - Post-transplant lymphoproliferative disorder (PTLD)
  - Nasopharyngeal CA
  - AIDS-associated "leiomyosarcoma" (pediatric)
Oncogene/Tumor suppressor mutations
- KIT/PDGFRA mutations in gastrointestinal stromal tumors
  - Target for treatment w/ Imatinib
- EGFR mutations in Lung CA
  - Adenocarcinomas in non-smokers
  - Response to treatment w/ Iressa
- PIK3CA and PTEN mutations
  - Activation of AKT pathway
  - Susceptibility to Rapamycin-type agents.

EGFR: in-frame 15bp Exon19 deletion
Direct sequencing: mixture of tumor and normal – lower mutant peaks

Oncogene/TSP mutations
- Oncogene mutations:
  - Activating mutations
  - Limited number of mutations/gene
  - Tests to detect specific mutations.
- Tumor Suppressor Gene mutations
  - Inactivating mutations
  - Many possible mutations
    - Occasionally hot-spots, related to gene structure and/or mutagen.

Tumor-specific translocations
- Translocations w/o fusion gene products
  - Dysregulation of gene expression, no fusion mRNA
  - Detect translocation by:
    - Classical cytogenetics, FISH, Southern Blotting or DNA PCR
    - DNA PCR for translocations
      - Requirement for recurrent breakpoints OR
      - Long range PCR.

Somatic mutation tests
- Recurrent/Known mutations:
  - Tests to detect specific sequences
    - Probes, restriction enzymes, sequence-specific primers, etc.
    - Can detect mutation in minority of cells.
    - Use of sequence-specific primers can enrich for mutant sequence.
- Tests for unknown mutations
  - Mutation scanning techniques
  - Direct Sequencing.

Translocations w/o Fusion RNA
<table>
<thead>
<tr>
<th>Tumor</th>
<th>Translocation</th>
<th>Activated Gene</th>
<th>Mechanism of Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-ALL/Burkitt</td>
<td>t(8;14)(q24;q32)</td>
<td>MYC</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>Large Cell</td>
<td>t(14;19)(q32;q13)</td>
<td>BCL6</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>t(11;14)(q13;q32)</td>
<td>Cyclin D1</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>Malignant</td>
<td>t(14;19)(q32;q13)</td>
<td>BCL2</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>B-cell</td>
<td>t(14;18)(q32;q21)</td>
<td>BCL2</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>Follicular B-</td>
<td>t(14;18)(q32;q13)</td>
<td>BCL2</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>cell lymphoma</td>
<td>t(14;18)(q32;q13)</td>
<td>BCL2</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>t(14;18)(q32;q13)</td>
<td>MYC</td>
<td>Relocation to light locus</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>t(14;18)(q32;q13)</td>
<td>TAL1</td>
<td>Relocation to light locus</td>
</tr>
</tbody>
</table>
DNA PCR for translocations: BCL2

- t(14;18)(q32;q21)

  - 14q32 breakpoints constant.
  - 18q21 breakpoints variable:

DNA PCR for translocations

- Variable breakpoints:
  - Not all translocations will be amplified by a limited primers set.
    - E.g., BCL2 – 60%, BCL1 – 50%
  - Need for "long range" PCR to increase detection
    - Technically difficult.
  - Needs high quality DNA – will not work on paraffin.

Translocations w/ chimeric products: solid tumors

Translocations w/ fusion RNA - Heme

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Translocation</th>
<th>Gene fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic myelogenous leukemia</td>
<td></td>
<td>BCR-ABL (p210)</td>
</tr>
<tr>
<td>Acute promyelocytic leukemia</td>
<td>t(15;17), t(11;17)(q23;q21), t(15;17)(q35;q31), t(11;17)(q13;q21), der(17)</td>
<td>PML-RAR, PLZF-RAR, NPM-RAR, NGMA-RAR, STAT5b-RAR</td>
</tr>
<tr>
<td>AML</td>
<td>t(8;21)(q22;q22)</td>
<td>AML1-ETO</td>
</tr>
<tr>
<td>AML and ALL (esp. infants and post-Rx)</td>
<td>t(11;23)</td>
<td>MLL - (~40 partners)</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma</td>
<td>t(2;5)(p23;q35)</td>
<td>PML-ALK</td>
</tr>
<tr>
<td>ALL</td>
<td>(9;22)</td>
<td>BCR-ABL (p190)</td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>t(1;18)</td>
<td>API2-MLT</td>
</tr>
</tbody>
</table>
Figure 13: Amplification plots of $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions of a BCR-ABL in-bcr-ABL positive RNA sample in a negative RNA sample.