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)
Molecular Diagnosis

“Nucleic Acid Testing”
- Use of specific sequence information in nucleic acids - DNA and RNA - for clinical management

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
Gain/Loss of Nucleic Acid

- Diagnosis of specific entities:
  - 1p/19q in oligodendrogliaoma; 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”.

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 or</td>
<td>Near diploid or</td>
<td>Near diploid or</td>
</tr>
<tr>
<td></td>
<td>near triploid</td>
<td>near tetraploid</td>
<td>near 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>TRK-A exp.</td>
<td>High</td>
<td>Low</td>
<td>Low or absent</td>
</tr>
<tr>
<td>TRK-B exp.</td>
<td>Low or truncated</td>
<td>Low or absent</td>
<td>High (full length)</td>
</tr>
<tr>
<td>TRK-C 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>95%</td>
<td>25–50%</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
Antigen Receptor Gene Rearrangement

Unique V-D-J rearrangements for each event.

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

Southern Blotting for Clonality

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

- **Southern**
  - Needs High MW DNA
  - 5-10 μg DNA
  - Labor intensive
  - Tech. demanding
  - At least 5% neoplastic cells
  - Detects most recombinations
  - Does not detect junctional diversity

- **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:**
  - Postr-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.

**Oncogene/TSP mutations**

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

EGFR: in-frame 15bp Exon19 deletion

Direct sequencing: mixture of tumor and normal – lower mutant peaks
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.

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 IgH locus</td>
</tr>
<tr>
<td>Large Cell Lymphoma</td>
<td>t(3;14)(q27;q32)</td>
<td>BCL6</td>
<td>Relocation to IgH locus</td>
</tr>
<tr>
<td>Mantle Cell Lymphoma</td>
<td>t(11;14)(q13;q32)</td>
<td>Cyclin D1</td>
<td>Relocation to IgH locus</td>
</tr>
<tr>
<td>Follicular B-cell lymphoma</td>
<td>t(14;18)(q32;q21)</td>
<td>BCL2</td>
<td>Relocation to IgH locus</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>t(8;14)(q24;q11)</td>
<td>MYC</td>
<td>Relocation to TCR α/δ locus</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>t(1;14)(p32;q11)</td>
<td>TAL1</td>
<td>Relocation to TCR α/δ 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 sets.
    - 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/ 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>t(9;22)</td>
<td>BCR-ABL(p210)</td>
</tr>
<tr>
<td>Acute promyelocytic leukemia</td>
<td>t(15;17); t(11;17)(q23;q21); t(5;17)(q35;q21); t(11;17)(q13;q21) der(17)</td>
<td>PML-RAR; PLZF-RAR; NPM-RAR; NUMA-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>11q23</td>
<td>MLL- (~40 partners)</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma (pediatric)</td>
<td>t(2;5)(p23;q35)</td>
<td>NPM-ALK</td>
</tr>
<tr>
<td>ALL</td>
<td>t(9;22)</td>
<td>BCR-ABL(p190)</td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>t(11;18)</td>
<td>API2-MLT</td>
</tr>
</tbody>
</table>

### Translocations w/chimeric products: solid tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Translocation</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewing’s Sarcoma</td>
<td>t(11;22); t(21;22); t(7;22); t(12;22)</td>
<td>EWS/ FLI1; EWS/ ERG; EWS/ ETV1; EWS/ ETV4</td>
</tr>
<tr>
<td>Alveolar Rhabdomyosarcoma</td>
<td>t(1;13); t(2;13)</td>
<td>PAX7/ FOXOA1; PAX3/ FOXOA1</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>t(X;18)</td>
<td>SYT/ SSX1</td>
</tr>
<tr>
<td>DSRCT</td>
<td>t(11;22)</td>
<td>EWS/ WT1</td>
</tr>
<tr>
<td>Myxoid/ round cell liposarcoma</td>
<td>t(12;22)</td>
<td>CHOP/ FUS</td>
</tr>
<tr>
<td>Clear cell sarcoma soft parts</td>
<td>t(12;22)</td>
<td>EWS.ATF-1</td>
</tr>
<tr>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>t(9;22)</td>
<td>EWS/ TEC</td>
</tr>
</tbody>
</table>
Figure 12  Schematic diagram of the BCR-ABL FG transcript covered by the RQ-PCR primer and probe set. For m-bcr, set: ENF402–ENP541–ENR561; and for M-bcr, set: ENF501–ENP541–ENR561. The number under the primers and probe refers to their 5’ nucleotide position in the normal gene transcript (see Tables 14 and 17). Relative frequency refers to the proportion of e1-a2 transcript among m-bcr variants and the proportion of b3-a2 and b2-a2 transcripts among M-bcr variants.
Figure 13  Amplification plots of $10^{-1}$, $10^{-3}$ and $10^{-4}$ dilutions of a \textit{BCR-ABL} m-bcr-positive RNA sample in a negative RNA sample.