Molecular Diagnosis

Basic Concepts, Genetic Alterations, Approaches to Detection, Interpretation, Clinical, Ethical and Legal Implications.

What is a “Genetic Test”?*

- Analysis of human
  - DNA, RNA, chromosomes, proteins, metabolites
- to detect heritable disease related
  - genotype, mutation, phenotype, or karyotype
- for clinical purposes.
- “Molecular” genetic test: DNA/RNA-based
  - Usu. PCR or related methodology, or Southern Blotting.

Human genome organization

- Human genome:
  - Total DNA content of cells
    - Nuclear genome – approx. 30,000 genes
    - Mitochondrial genome – 37 genes.
- Nuclear genome:
  - 24 linear double-stranded DNA molecules.
    - 1.5% coding
    - 3% non-coding highly conserved in mammals
    - 45% transposon-based repeats!
    - 6.6% heterochromatin repeats
    - 44% other non-conserved

Coding DNA

- 90-95% encode mRNA – polypeptides.
- 5-10% - RNA genes.

DNA sequence families (related coding sequences)

- Arise from gene duplication
- Clustered (e.g., V-family genes) or dispersed

Pseudogenes/gene-fragments

- Non-functional gene-related segments
- May contain introns (duplication events) or lack them
  (“processed pseudogenes” - retrotransposition events.)
- Estimated 20,000 pseudogenes in human genome.

Nuclear genome organization

Mitochondrial genome

- 16,569bp, 44% GC
  - “H” strand – rich in G; “L” strand – rich in C.
- 37 genes – 28 encoded on “H”, 9 on “L”
  - 22 tRNA, 2 rRNA
  - 13 polypeptide genes
    - 13 of > 80 subunits of respiratory complexes of oxidative phosphorylation system.
- Variable number per cell.
- “Heteroplasmy”
Mitochondrial Genetic Code

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nuclear Code</th>
<th>Mitochondrial code</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGA</td>
<td>Arg</td>
<td>Stop</td>
</tr>
<tr>
<td>AGG</td>
<td>Arg</td>
<td>Stop</td>
</tr>
<tr>
<td>UGA</td>
<td>Stop</td>
<td>Trp</td>
</tr>
<tr>
<td>AUA</td>
<td>Ile</td>
<td>Met</td>
</tr>
</tbody>
</table>

Types of mutations and their consequences

Implications for Molecular genetic diagnosis

Mutations: functional vs. phenotypic effect

- Phenotypic effect of mutation
  - Effect on phenotype of individual with mutation.
- Functional effect:
  - No change in gene function.
    - E.g., point mutation w/ no AA change.
  - Loss of function:
    - Gene product with reduced or absent function.
  - Gain of function
    - Mutant gene product does something abnormal.

Loss of Function Mutations

- Usually Recessive Phenotypes
  - Dominant phenotype w/
    - Haploinsufficiency
      - E.g., BMPR-2 mutations in Primary Pulmonary hypertension
    - Dominant negative effect
      - E.g., Fibrillin-1 mutations in Marfan Syndrome.
    - Hereditary Cancer Syndromes
      - Somatic loss of second allele (“second hit”).
- Many mutations in gene w/ similar phenotype.
  - Point mutations, frame-shift mutations and deletions with similar phenotypes.

Loss of Function mutations

<table>
<thead>
<tr>
<th>Change</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire gene deletion</td>
<td>α-thalassemia</td>
</tr>
<tr>
<td>Partial gene deletion</td>
<td>60% of DMD</td>
</tr>
<tr>
<td>Insertions</td>
<td>LINE-1 insertion in F8</td>
</tr>
<tr>
<td>Translocations</td>
<td>Women w/ DMD</td>
</tr>
<tr>
<td>Inversion</td>
<td>F8 inversion</td>
</tr>
<tr>
<td>Promoter mutation</td>
<td>β-globin -29 A&gt;G</td>
</tr>
<tr>
<td>Promoter methylation</td>
<td>Many cancers</td>
</tr>
<tr>
<td>Poly-A site mutation</td>
<td>α-globin AATAAA&gt;AATAGA</td>
</tr>
</tbody>
</table>
Loss of Function mutations

<table>
<thead>
<tr>
<th>Change</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsense mediated RNA decay</td>
<td>Beta-globin Q39X</td>
</tr>
<tr>
<td>Splice donor loss</td>
<td>PAX3 451+1 G&gt;T</td>
</tr>
<tr>
<td>Splice acceptor loss</td>
<td>PAX3 452-2 A&gt;G</td>
</tr>
<tr>
<td>A Exonic splicing enhancer</td>
<td>SMN2 Exon7 change</td>
</tr>
<tr>
<td>Activate cryptic splice site</td>
<td>CFTR 3849 +10kb C&gt;T</td>
</tr>
<tr>
<td>Frame-shift mutation</td>
<td>BRCA1 185delAG</td>
</tr>
<tr>
<td>Nonsense mutation</td>
<td>PAX3 Q254X</td>
</tr>
<tr>
<td>Missense mutation</td>
<td>HFE C282Y</td>
</tr>
</tbody>
</table>

Gain of Function Mutations

- Usually dominant phenotypes
- Only few (or one) mutation(s) per gene with gain of function effect

Gain of Function Mutations

- Factor V Leiden
  - Arg506Gln.
  - Resistance to inactivation by activated Protein C.
  - Increased thrombotic risk

Gain of Function Mutations

- Fibroblast Growth Factor Receptor 3 (FGF3)
  - Receptor tyrosine kinase, activated by binding FGF, to start signaling cascade.
  - Gly390Arg (1138 G>A):
    - “Mildly” increased activity (requires FGF binding)
    - ACHONDROPLASIA.
  - Y373C, R248C, and S249C
    - Create extracellular cysteine
    - Ligand independent binding: “greatly increased” activity.
    - Severe phenotype: neonatal death (“Thanatophoric Dysplasia.”)

Nucleic-acid alterations targeted in molecular diagnosis

- Molecular Genetics:
  - Recurrent mutations
  - “Private” mutations
    - Gross alterations vs. “smaller” alterations
    - Coding region vs non-coding region alterations
  - Trinucleotide repeat alterations.
    - Very large repeats defy amplification methods.
  - Mitochondrial DNA alterations.
    - “heteroplasmy”.

Samples for Molecular Genetic Diagnosis

- DNA Testing: Any nucleated cells.
  - Blood samples; mouthwashes or buccal scrapes; CVS samples; 1-2 cells from 8 cell stage; hair; semen; archived pathological specimens (dead patients); “Guthrie cards” (dried blood spots).
- RNA Testing: Cells which express the transcript.
RNA vs. DNA

- DNA:
  - Stable; can be directly amplified w/PCR; easy to work with.
  - Obtainable from any nucleated cells.
  - Contains introns:
    - Need to amplify each exon separately (w/ large introns)
    - Introns not tested – cryptic splice sites may be missed.
  - Breakpoints variable:
    - Problem w/ PCR-based methods.

- RNA:
  - No introns: RT-PCR in fewer segments.
  - Effects of mutations seen in mRNA
    - Cryptic splice sites; Gross rearrangements
    - RNA fusions less variable than DNA breakpoints
  - Difficult to work w/:
    - Need for RT step
    - Ubiquitous Ribonucleases.
  - Need for cells w/c express RNA
  - Not all rearrangements w/ gene fusion
  - Nonsense-mediated decay.

Mutation Detection Tests

- Tests for known mutations
  - One mutation (e.g., Factor V Leiden)
  - A few mutations (e.g., CF 23 mutations plus 2 polymorphisms)
  - Many mutations

- Tests for known & Unknown mutations
  - Mutation “scanning” tests
    - Detect presence, not identity of variant
  - Sequencing (DNA or RNA)
    - Presence & identity of variant.

Tests for known mutations

- Most use some form of target amplification
  - E.g. Polymerase chain reaction
  - Either a probe, or a restriction enzyme is used to distinguish normal from mutant sequence.
  - Results, shown as presence or absence of the specific mutation.
  - No information on presence or absence of other mutations in gene.

Mutation Scanning Methods

- Mutation in family not known.
- Scan multiple segments of one or more genes for mutations in.
  - Exons
  - Introns, introns, splice sites, promoters, enhancers, “locus control region”, etc.
- Specific strategy determined by clinical syndrome/test purpose.
“Physical”/screening methods

- Physical properties of amplified gene segments
  - Denaturation profile, electrophoretic mobility, etc.
  - SSCP (single strand conformation polymorphism)
  - DGGE (Denaturing gradient gel electrophoresis)
  - DHPLC (denaturing high performance liquid chromatography)
  - Cleavage fragment length polymorphisms
  - Heteroduplex analysis
  - Dideoxy fingerprinting.

“Physical”/screening methods

- Sensitivity determined by specific mutation
- Need for multiple conditions
- One datapoint per gene segment evaluated
- Screen for presence not identity of mutation.

Mutation Scanning Methods

- Direct Sequencing
  - Screen presence and identity of mutation
  - Bidirectional sequencing
  - 2 datapoints per base sequenced
  - DNA sequencing
    - Usual multiple exons tested.
    - Splice-site mutations may be missed, especially mutations deep in large exons.
  - RNA sequencing
    - Need for cells w/ express gene
    - “Nonsense mediated decay”
    - RNA more labile

Testing Strategies.

- Single gene disease w/ only recurrent mutations:
  - e.g., Multiple Endocrine Neoplasia-2 (MEN-2)
    - Activating (gain of function) mutations in RET proto-oncogene.
    - 55kb gene w/21 Exons.
    - Mutations limited to: 13 codons on exons 10, 11, 13, 14, 15, 16.
    - Test for specific mutations.
    - Positive and negative results:
      - High positive and negative predictive values.

Testing Strategies.

- Single gene ds w/recurrent and private mutations
  - e.g., CFTR, thalassemias.
  - Test for “ethnic” recurrent mutation(s)
  - If positive, significance known
  - If negative, and index case or relative, perform “mutation scanning” test.
    - if positive, probably significant, family testing may help.
    - if negative, significance depends on whether index case or relative.
Recurrent mutations: Cystic Fibrosis

- **CF**: AR; disease when 2 mutated CFTR alleles.
  - 1:3,300 Caucasians;
  - 1 in 9,500 Hispanics;
  - <1 in 50,000 Native Africans and Asians (Af.Am. 1:15K, As. Am. 1:32K)
- **NIH consensus statement**:
  - Offer testing to all planning pregnancy.

**BUT**: 900 CFTR MUTATIONS AND COUNTING!!!!!!

**Solution**:
- Test for most common mutations (currently 25)
  - i.e., test for recurrent mutations w/c will detect most cases in population.

### CFTR: INCIDENCE, CARRIER, MUTATION RATES: BY POPULATION

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Carrier freq.</th>
<th>%ΔF508</th>
<th>% other &quot;common&quot;</th>
<th>% group-specific</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>1:3,300</td>
<td>1/29</td>
<td>70</td>
<td>13</td>
<td>80-90%</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>1/8-9000</td>
<td>1/46</td>
<td>46</td>
<td>11</td>
<td>57%</td>
<td></td>
</tr>
<tr>
<td>Ashkenazi</td>
<td>1/3,300</td>
<td>1/29</td>
<td>30</td>
<td>67</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>1:1500-2970</td>
<td>1/40</td>
<td>0</td>
<td>25</td>
<td>69</td>
<td>94%</td>
</tr>
<tr>
<td>African American</td>
<td>1:15,300</td>
<td>1/60-65</td>
<td>48</td>
<td>4</td>
<td>23</td>
<td>75%</td>
</tr>
<tr>
<td>Asian American</td>
<td>1:32,100</td>
<td>1/90</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>30%</td>
</tr>
</tbody>
</table>

**Negative results, Screening**

- **Caucasian Couple, no family hx. both test (-):**
  - Carrier rate = 0.04
  - Population incidence ~ 0.0016*0.25 = 1 in 2500
  - “Residual Risk” = 0.00249 (94% detection rate)
    - probability of affected child ~ 1 in 645,000
- **Hispanic couple:**
  - Carrier rate = 0.022
  - Population incidence ~ 1 in 8-9000
  - “Residual Risk” = 0.00958
    - probability of affected child ~ 1 in 43,584

**Negative results: + family history**

- **Asian Couple**:
  - Carrier rate = 0.011
    - probability of affected child ~ 1: 32,000
  - “Residual Risk” = .0077
    - probability of affected child ~ 67500
- **Caucasian Couple**
  - each w/ sibling with CF
    - (Mutation in sibling not known).
    - (Prior Probability of each parent being a carrier = 2/3).
  - Both test negative for the 25 mutations.
    - Probability of being a carrier (each parent) = 0.168 .
    - Probability of affected child = 1 in 140
Negative results: + family history

- Hispanic Couple w/ same history and results:
  - Probability or being carrier = (0.467)
  - probability of an affected child = 1 in 20!

- Asian couple w/ same hx and results:
  - probability of affected child 1 in 12!

Expanded trinucleotide repeats

- Southern Blotting Methods
  - Gold Standard
  - Labor intensive, need for high quality DNA

- PCR-based Methods
  - Rapid
  - Amplification failure of very long repeats.

Expanded Repeats-Huntington Disease

<table>
<thead>
<tr>
<th>Normal</th>
<th>(CAG)3-26</th>
<th>(CAG)27-55</th>
<th>(CAG)35-41</th>
<th>(CAG)42-121</th>
</tr>
</thead>
<tbody>
<tr>
<td>At risk for expansion</td>
<td>Variable penetrance</td>
<td>Affected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Test Strategy: “Private” mutations

- DNA sequencing of “entire” gene(s):
  - Usually only exons and adjacent introns.
  - Will not detect:
    - Mutations deep in introns causing alternative splicing.
    - Large deletions/rearrangements.

- RNA sequencing:
  - Detect abnormal transcripts:
    - Splice mutations; large deletions, rearrangements.
  - Will not detect whole gene deletions!

Test Strategy: “Private” mutations

- Tests for large deletions:
  - In-situ hybridization, Southern Blotting, "quantitative" PCR and related methods ("multiplex probe ligation-dependent amplification" – “MLPA”)

How to Interpret a Test Result

- Pathogenic Mutation
- No Variation
- Variation of unknown clinical significance
**Result Interpretation:**

- Previously reported mutation
  - Known to be cause of disorder
  - Known to be “neutral variation”
- New mutation:
  - Type likely to be assoc. w/disorder
    - frame-shift mutation, start “ATG” mutation, “Stop codon” nonsense mutation, nonsense mutation, splice-junction mutation, non-conservative missense in active site,
  - Type likely to be “neutral”
    - e.g., no change in amino acid, and not cryptic splice site
  - Type w/c may or may not be assoc. w/ disorder
    - E.g., non-conservative missense mutation, in region not known to be active site, etc.

**RESULT INTERPRETATION**

- Two mutations (Recessive Disorders)
  - Test parents to ensure two mutations in trans (separate alleles) not in cis (same allele).
- No mutation detected.
  - Residual risk depends on individual gene
    - some genes - mainly point mutations, easily detected.
    - Other genes: deletions, rearrangements, intronic alterations, etc., common (e.g., Neurofibromatosis1, BMPR2 - need special tests e.g., tests for gene dosage, etc.).

**Genetic Testing: Additional Considerations**

- Screening vs Genetic testing of “index” case
  - With “index” case, it is known that tested individual has clinical disease; only value of negative test is that you know that it cannot be used to screen relatives.
- Locus heterogeneity:
  - Multiple genes causing same syndrome
- Variable “penetrance”
  - May or may not depend on specific mutation.
- Variable expressivity
  - Variable severity of disease.
  - May or may not depend on specific mutation

**Benefits Vs. Risk of Testing:**

- Availability of treatment/prevention
- Pre-clinical manifestations.
- Discrimination:
  - Insurance
  - Employment
  - Confidentiality

**Factors affecting utility of genetic testing**

- Increased Utility
  - High morbidity/mortality of disease
  - Effective but imperfect Rx
  - High predictive power test (high penetrance)
  - Screening/surveillance expensive/difficult
  - Preventive measures expensive or associated with adverse effects
- Decreased utility
  - Low morbidity/mortality of disease
  - Highly effective and acceptable Rx (i.e., can wait for clinical disease)
  - Poor predictive power of genetic test (low penetrance)
  - Screening simple/needed regardless of mutation status
  - Preventive measures inexpensive, efficacious, and highly acceptable - e.g., folate supplementation.

Genetic Testing: Additional Considerations

- Ethics
  - implications for patients and relatives.
    - e.g., identical twins; siblings;
    - paternity issues -
- Legal issues
  - New York State Civil Right Law:
    - Need for informed consent
      - Genetic testing only (not phenotypic testing)
      - Standards for informed consent in civil rights law, section 79-l
        [http://assembly.state.ny.us/leg/?id=17&a=12].