Molecular Diagnosis

of Inherited Diseases

TOPICS

• Definition and uses of genetic tests
• Tests for “Gene level” alterations
  – Structure of the gene
  – Types of mutations
  – Tests for “recurrent” mutations
  – “Mutation Scanning” tests
  – Interpretation of positive and negative results
  – Ethical and additional considerations in testing
Introduction

- Define A Genetic Test
- Discuss settings in which one may use genetic tests.
- List types of genetic alterations one may test for.
- Factors which affect test choice.

Molecular Genetic Tests

- Genetic test:
  - Analysis of human
  - DNA, RNA, chromosomes, proteins, metabolites
  - to detect heritable disease-related
  - genotype, mutation, phenotype, or karyotype
  - for clinical purposes.
Genetic Disease

- All disease?
- Types of genetic diseases:
  - Chromosomal disorders.
  - Contiguous gene syndromes.
  - Single gene disorders.
    - Distinct phenotype.
    - Different genes same phenotype.
  - “Multigene” disorders.

Genetic diagnosis: “Purpose”

- Diagnostic Testing
  - Symptomatic individual
- Screening
  - Entire population or high risk group (e.g., CF, “Ashkenazi panel”)
- Presymptomatic Testing
  -Usu. Positive fam hx., e.g., Huntington’s.
- Prenatal testing
  - Chromosomal disorders, single gene disorders, RhD.
Genetic diagnosis: "Purpose"

- Preimplantation Genetic Diagnosis
  - Test for multiple possible disorders on one or two cells!
- Pharmacogenetic testing
  - e.g., TPMT & thiopurine drugs
- Testing for susceptibility to environmental agents
  - e.g., Paraoxonase & organophosphate toxicity
  - ABUSE: (e.g., genetic testing of railroad workers w/ carpal tunnel syndrome!)

Test Choice

- Cost
  - Material costs, Personnel costs.
    - Automated tests have lower personnel costs per test.
  - Reduce overall costs by “multiplexing”
    - E.g., use of arrays to test multiple genes, chromosomes, etc.
- Sample requirements
  - E.g. conventional cytogenetics - live cells
- Turnaround time
  - E.g. prenatal, PGD – need for rapid turnaround.
Test Choice

- **Test Validity:**
  - Sensitivity, Specificity
  - Analytical vs. Clinical validity
  - Analytical validity
    - Correctly identifies presence/absence of *mutation*
  - Clinical Validity:
    - Correctly identifies presence/absence/risk of *disease.*
    - Positive & negative predictive values

Test Choice

- **Type of genetic Δ to be detected**
  - Chromosomal abnormality
    - Conventional cytogenetics; FISH; CGH, etc
  - Gain or loss of genetic material:
    - Conventional cytogenetics; FISH; CGH, etc
  - Known mutation(s) in one or more genes.
    - Many methods: e.g., PCR-RFLP, SSP, probes, etc.
  - Unknown mutation in one or more genes.
    - “Mutation Scanning” Techniques (e.g., sequencing, “SSCP”, dHPLC), etc.
    - Direct DNA/RNA sequencing
Gene Level Alterations

Topics

- Structure of genes.
- Types of mutations, and potential consequences.
- Types of tests for “known” or “recurrent” mutations (inc. expanded repeats).
  - interpretation of positive and negative tests for “known” mutations.
- Tests for “unknown” mutations.
  - interpretation of positive and negative tests for “unknown” mutations.
Structure of Genes

Types of mutations
### Point Mutations

<table>
<thead>
<tr>
<th>Missense</th>
<th>Nonsense</th>
<th>Silent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td>TTC</td>
<td>ATC</td>
</tr>
<tr>
<td>Leu</td>
<td>Phe</td>
<td>Leu</td>
</tr>
<tr>
<td>TTC</td>
<td>AGC</td>
<td>TTC</td>
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<tr>
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<tr>
<td>GAG</td>
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</tr>
<tr>
<td>Glu</td>
<td>Leu</td>
<td>CTA</td>
</tr>
<tr>
<td>CTA</td>
<td>TAT</td>
<td>Leu</td>
</tr>
<tr>
<td>Leu</td>
<td>Tyr</td>
<td>Tyr</td>
</tr>
</tbody>
</table>

#### Missense Mutations

- Depending upon specific AA change
  - Loss of function:
    - e.g., Hb S, Hemochromatosis
  - "Gain of Function":
    - e.g., Factor V Leiden
  - No functional effect:
    - e.g., KVLQT1 P448R
Missense mutations

When is a missense mutation significant?

- known structural and functional domain
- evolutionarily conserved residue
- independent occurrence in unrelated patients
- absent in large control sample
- novel appearance & cosegregation w/disease phenotype in pedigree
- In vitro loss of function
- restoration of function by WT protein.

Sickle Cell Anemia Mutation

\[
\begin{array}{c}
\text{MstI} \\
\downarrow 1.15 \text{ kb} \\
\text{MstII} \\
\downarrow \\
\text{Hb } \beta^A \\
\text{Glu} \\
\downarrow 1.35 \text{ kb} \\
\text{MstII} \\
\downarrow \\
\text{Hb } \beta^s \\
\text{Val} \\
\end{array}
\]

\begin{align*}
\ldots \text{CCT GAG GAG} \ldots & (\text{Hb } \beta^A) \\
\ldots \text{CCT GTG GAG} \ldots & (\text{Hb } \beta^S) \\
\text{CCT NAG G} & (\text{MstII}) \\
\ldots \text{CCT AAG GAG} \ldots & (\text{Hb } \beta^C)
\end{align*}
Nonsense Mutations

- Amino Acid codon to “Stop”
- Three stop codons
  - UAA, UAG, UGA
- Truncated protein
  - Protein truncation test
- E.g., Beta° Thalassemia in Sardinia
  - Codon 24, CAG to TAG

Deletions

```plaintext
CATGTAGGCAAT
```

```plaintext
CATGTAGCAAT
```

```plaintext
CATGTAGCAAT
```
Deletions

- Complete/partial gene deletions
  - Duchenne Muscular Dystrophy
  - Alpha thalassemia
- Multiple genes ("contiguous gene syndromes")
  - DiGeorge Syndrome chr 22q11.2
  - TSC2-PKD1 Chr. 16p13
  - WAGR syndrome 11p112-13
Splice Junction Mutations

- GT/AG rule
  - AAGGTAAAGT...
- Loss of splice site
  - intron incorporated in mRNA
- Creation of novel splice sites
  - >100 mutations
    - e.g., Hemoglobin E
- Hemoglobin E
- Missense mutation and splice site error
- Both normal and new splice site use
  • Hemoglobinopathy (missense) AND thalassemia (reduced Hb) features

Hemoglobin E (Glu26Lys)

60%

40%

Insertions

- Tay Sachs Disease
  - 4bp insertion in Ashkenazi Jews
- Hemophilia A
  - L1 insertion in FVIII gene (1% of patients)
Frame-Shift Mutations

- Codon = 3 bp
- insertion/deletion not multiple of 3bp
  - Change of reading frame - entire protein altered.
  - e.g., Tay Sachs 4 bp insertion, BRCA1 185 delAG, BRCA2 6174delT, etc.
  - blood group O (1 bp deletion)

Other mutations

- Cap-site Mutants
- Mutations in initiation codons
- Creation of a new initiation codon
- Mutations in termination codons
- Polyadenylation/cleavage signal mutations.
Unstable Trinucleotide Repeats

- Expansion tandem repeats of trinucleotides.
  - promoter/5’UTR
    - Fragile X Syndrome (CGG)n 5’UT
  - Exon
    - Huntington’s syndrome (CAG)n polyglutamine
    - SCA type 1 (CAG)n polyglutamine
  - Intron
    - Friedrick’s Ataxia (GAA)n intron
  - 3’UTR
    - Myotonic dystrophy (CTG)n 3’UT

![Bar chart showing different types of genetic alterations](chart.png)
“Known” Mutations

- **“Recurrent” Mutations**
  - Same mutation in multiple unrelated families
  - Single mutation assoc. w/phenotype
    - E.g., Sickle cell disease; Factor V Leiden, Hb. C
  - Limited # of mutations in gene assoc. w/phenotype.
    - E.g., Hemochromatosis C282Y and H63D; MEN-2, Achondroplasia, etc.
  - Ethnic group-specific mutations
    - E.g., BRCA1, BRCA2, “Ashkenazi” panel, CF.
  - Known mutation in family.

Tests for “known” mutations

- Many rapid, sensitive/specific methods available.
- Test choice - laboratory preference
  - workflow, available equipment, kit availability, patent issues, etc.).
- Detect
  - heterozygotes (one mutant allele)
  - compound heterozygotes (two different mutations)
  - Homozygotes (two alleles with same mutation).
Recurrent Mutations

Methods
- PCR-RFLP
- Allele-specific probes/primers
- Direct sequencing/“Minisequencing”/Pyrosequencing.
- Molecular Beacons/TaqMan probes.
- Oligonucleotide ligation assay.
- Mass spectroscopy-based methods.

Tests for recurrent mutations

Choice of mutation tested for
- Clinical syndrome
  - E.g., Thrombosis – Factor V Leiden and Prothrombin mutation
  - Medullary thyroid carcinoma – MEN2 mutations etc.,
  - Hemochromatosis, test for HFE mutations.
- Ethnicity
  - E.g., Ashkenazi Panel.
- Family History.
Tests for recurrent mutations

**Results:**
- Mutation tested for either not present, heterozygous, or homozygous.

**Positive results**
- Unambiguous
- Technical false positive rare (*most* methods)
- Positive predictive value, penetrance, etc. usu known
  - (Exceptions: HFE mutations – penetrance not agreed upon; and family-specific mutation).

**Negative Result:**
- “Residual risk” [for mutation, not disease] determined by two factors:
  - Risk of having mutation prior to testing
  - Sensitivity of mutation panel for patient’s ethnic group.
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.

Recurrent mutations: Cystic Fibrosis

- 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

<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></td>
<td>80-90%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1/8-9000</td>
<td>1/46</td>
<td>46</td>
<td>11</td>
<td></td>
<td>57%</td>
</tr>
<tr>
<td>Ashkenazi</td>
<td>1:3,300</td>
<td>1/29</td>
<td>30</td>
<td>67</td>
<td></td>
<td>97%</td>
</tr>
<tr>
<td>Native Am.</td>
<td>1:1500-3970</td>
<td>0</td>
<td></td>
<td>25</td>
<td>69</td>
<td>94%</td>
</tr>
<tr>
<td>African Am.</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 Am.</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>

Caucasian Couple, no family hx. both test (-):
- Carrier rate = 0.04
  - Population incidence = 0.0016*0.25 = 1 in 2500
- "Residual Risk" = 0.04*(1-.94) = 0.0024 (Each Parent)
  - probability of affected child = 0.00000576*0.25~ 1 in 69000

Hispanic couple:
- Carrier rate = 0.022
  - Population incidence ~ 1 in 8-9000
- "Residual Risk" = 0.022*(1-.57) = .00946
  - probability of affected child ~ 1 in 45,000
Asian Couple:
- Carrier rate = 0.011
  - probability of affected child ~ 1: 32,000
- “Residual Risk” = 0.011*(1-0.3) = 0.0077
  - probability of affected child ~ 67500

CFTR Negative results: Screening

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

(CAG)$_{10-26}$  (CAG)$_{27-35}$  (CAG)$_{35-41}$  (CAG)$_{42-121}$

Normal  At risk for expansion  Variable penetrance  Affected

Tests for unknown mutations

“Mutation Scanning Methods”
Mutation Scanning Methods

- Mutation in family not known.
- No recurrent mutations
- Look for mutations in
  - Exons
  - Introns,
  - splice sites,
  - promoters,
  - enhancers,
  - “locus control region”, etc.
- Of one or more genes.

Mutation Scanning Methods

- Ideal mutation scanning method:
  - Screen large DNA sequence
  - 100% sensitivity and specificity
  - Unambiguously define mutation.
  - Minimum # of steps
  - High throughput
  - No special equipment
  - No dangerous reagents
- No such method
  - Compromise
**Mutation Scanning Methods**

- Detect difference in physical properties of normal and mutant DNA.
- Directly sequence genomic DNA.
- Reverse transcribe RNA and sequence cDNA.
- Test properties of translated protein - using DNA or RNA as starting material.

**Screening “physical properties”**
- Test for altered denaturation profile, or electrophoretic mobility.
  - e.g., SSCP, DGGE, DHPLC, Cleavase fragment length polymorphisms, heteroduplex analysis, dideoxy fingerprinting.
  - Sensitivity varies for different genes/mutations.
  - Need to use multiple conditions.
  - One datapoint per gene segment evaluated.
- Screen for presence, not identity of mutation.
Mutation Scanning Methods

- **Direct Sequencing**
  - Screen for presence and identity of mutation
  - Genomic DNA sequencing
    - Bidirectional sequencing (both strands)
    - **Two datapoints per base evaluated**
    - usu. multiple exons tested
    - splice-site mutations may be missed
  - cDNA sequencing
    - Use RNA from cells w/c express gene (no introns)
    - Splicing alterations detected
    - Caution: “nonsense mediated decay”
      - RNA w/ early nonsense mutation is degraded by cells
      - Only normal RNA will be sequenced

Direct Sequencing Methods

- **Automated fluorescent sequencing**
  - widely available
  - DNA segment amplified by PCR
  - PCR product used as template for “cycle sequencing”
  - need to inspect electropherograms
    - verify “base calling”, heterozygous bases
How to Interpret a Test Result

- Pathogenic Mutation
- No Variation
- Variation of unknown clinical significance
Mutation Scanning Tests

- **Mutation detected**
  - 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”
        misense 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.

- **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.).
Molecular Genetic Testing

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 and mortality of the disease
- Effective but imperfect treatment
- High predictive power of genetic test (high penetrance)
- High cost or onerous nature of screening and surveillance methods
- Preventive measures that are expensive or associated with adverse effects

**Decreased utility**
- Low morbidity and mortality of disease
- Highly effective and acceptable treatment (i.e., no harm is done by waiting for clinical disease to treat patient)
- Poor predictive power of the genetic test (low penetrance)
- Availability of inexpensive, acceptable, and effective surveillance methods (or need for surveillance whether or not one has increased genetic risk)
- Preventive measures that are 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/?cl=17&a=12].