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

- 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

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

Gene Level Alterations

- 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.

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
**Structure of Genes**

- **UTR**
  - 5' UTR
  - 3' UTR
- **Exon**
- **Intron**
- **Splice sites**
- **Promoter**
- **Enhancer**

**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

**Types of mutations**

- **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.

**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>TTC</td>
</tr>
<tr>
<td>TTC</td>
<td>AGC</td>
<td>AGC</td>
</tr>
<tr>
<td>TGC</td>
<td>TGC</td>
<td>TGC</td>
</tr>
<tr>
<td>GAG</td>
<td>GAG</td>
<td>GAG</td>
</tr>
<tr>
<td>CTA</td>
<td>CTA</td>
<td>CTA</td>
</tr>
<tr>
<td>TAT</td>
<td>TAT</td>
<td>TAT</td>
</tr>
</tbody>
</table>

**Sickle Cell Anemia Mutation**

- **Hb S**
- **Hb F**
- **Glu**
- **Leu**
- **Stop**
- **Cys**
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

- 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
  - AAGGTAAGT.../... YYYYYYYYYYNCGAG
- 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)

- GGT GGT GAG GCC BetaA
- GGT GGT AAG GCC BetaE

Other mutations

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

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 6174delIT, etc.
  - Blood group O (1 bp deletion)

Unstable Trinucleotide Repeats

- Expansion tandem repeats of trinucleotides.
  - Promoter/5'UTR
    - Fragile X Syndrome (CGG)n 5'UTR
  - Exon
    - Huntington's syndrome (CAG)n polyglutamine
    - SCA type 1 (CAG)n polyglutamine
  - Intron
    - Friedreich's Ataxia (GAA)n intron
  - 3'UTR
    - Myotonic dystrophy (CTG)n 3'UTR
**“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).

**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).

**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
  - 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.

CFTR 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” = 0.04*(1-.96) = 0.0024
  - Probability of affected child = 0.000000576*0.25~ 1 in 6900
- Hispanic couple:
  - Carrier rate = 0.022
    - Population incidence = 1 in 8000
    - “Residual Risk” = 0.022*(1-.57) = .00946
    - Probability of affected child = 1 in 45,000

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 Negative results: Screening

- Asian Couple:
  - Carrier rate = 0.011
    - Probability of affected child ~ 1: 32,000
    - “Residual Risk” = 0.011*(1-0.3) = .0077
    - Probability of affected child ~ 67500

CFTR: Incidence, Carrier, Mutation Rates

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Carrier freq.</th>
<th>%ΔF508</th>
<th>% other “common”</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>13</td>
<td>18</td>
<td>85</td>
<td>95%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1:8-9000</td>
<td>1/46</td>
<td>46</td>
<td>14</td>
<td>13</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>97</td>
<td>97%</td>
</tr>
<tr>
<td>Native Am.</td>
<td>1:1500 – 570</td>
<td>0</td>
<td>25</td>
<td>69</td>
<td>94</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>10</td>
<td>0</td>
<td>0</td>
<td>30%</td>
</tr>
</tbody>
</table>

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)_{36-41}  (CAG)_{42-121}

Normal  At risk for expansion  Variable penetrance  Affected

Tests for unknown mutations

“Mutation Scanning Methods”

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.

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

- 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
  - RNA sequencing
  - Use RNA from cells w/ 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

- **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.

Mutation Scanning Tests

- **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

Additional considerations

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

<table>
<thead>
<tr>
<th>Increased utility</th>
<th>Decreased utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>High morbidity and mortality of disease</td>
<td>Low morbidity and mortality of disease</td>
</tr>
<tr>
<td>Effective but imperfect treatment</td>
<td>Highly effective and acceptable treatment (i.e., no harm is done by waiting for clinical disease to treat patient)</td>
</tr>
<tr>
<td>High predictive power of genetic test (high penetrance)</td>
<td>Poor predictive power of the genetic test (low penetrance)</td>
</tr>
<tr>
<td>High cost or onerous nature of screening methods</td>
<td>Availability of inexpensive, acceptable, and effective surveillance methods (or need for surveillance whether or not one has increased genetic risk)</td>
</tr>
<tr>
<td>Preventive measures that are expensive or associated with adverse effects</td>
<td>Preventive measures that are inexpensive, efficacious, and highly acceptable - e.g., folate supplementation.</td>
</tr>
</tbody>
</table>


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].