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

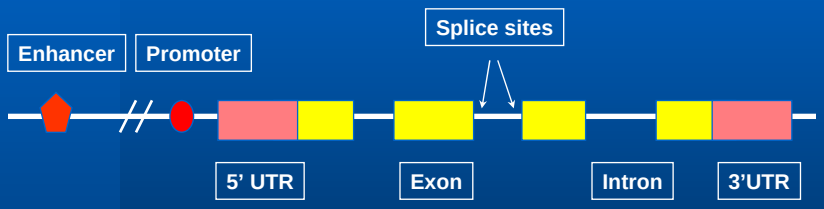
- Type of genetic Δ to be detected
  - Chromosomal abnormality
    - Conventional cytogenetics; FISH; CGH, etc
  - Gain or loss of genetic material:
    - Conventional cytogenetics; FISH; CGH, etc
  - Known mutation(s) in one or more genes.
    - Many methods: e.g., PCR-RFLP, SSP, probes, etc.
  - Unknown 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

	ATC	TTC	AGC	TGC	GAG	CTA	TAT
	Leu	Phe	Ser	Cys	Glu	Leu	Tyr
Missense	ATC	TT**A**	AGC	TGC	GAG	CTA	TAT
	Leu	**Leu**	Ser	Cys	Glu	Leu	Tyr
Nonsense	ATC	TTC	AGC	TG**A**	GAG	CTA	TAT
	Leu	Phe	Ser	**Stop**			
Silent	ATC	TTC	AGC	TGC	GAG	CT**G**	TAT
	Leu	Phe	Ser	Cys	Glu	Leu	Tyr

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

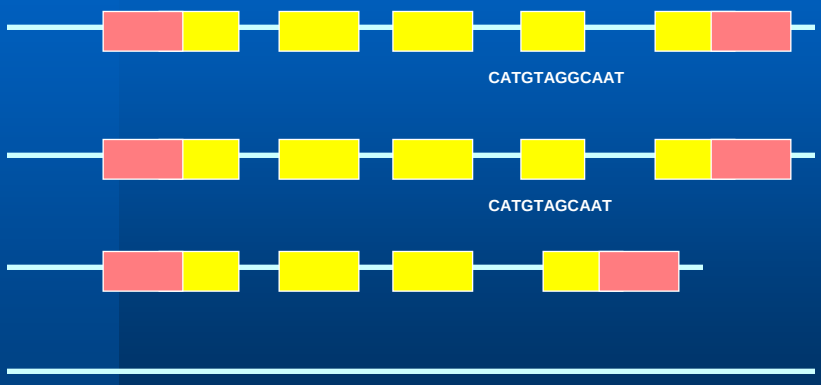
- **When is a missense mutation significant?**
  - known structural and functional domain
  - evolutionarily conserved residue
  - independent occurrence in unrelated patients
  - absent in large control sample
  - novel appearance & cosegregation w/disease phenotype in pedigreee
  - In vitro loss of function
  - restoration of function by WT protein.

Sickle Cell Anemia Mutation

Nonsense Mutations

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

Deletions



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**

wpod?am fkw cu.gjhklf four sjckfo qw score
and void m$%d jkkk yp@mvjckd fkkseo
cbqw,oiwjfm du seven years ago dllfkk*wqm
fkkd xmmcnfyruuei our skkdj$fmvjkjdfk&%wo
qppalfdkkf qaq.d eiidty forefathers brought jjd
qpoooekfjg vbzxx dsg forth a

wpod?am fkw cu.gjhklf four sjckfo qw score
and void m$%d jkkk yp@mvjckd fkkseo
cbqw,oiwjfm du seven years ago dllfkk*wqm
fkkd xmmcnfyruuei our skkdj$fmvjkjdfk&%wo
qppalfdkkf qaq.d eiidty forefathers brought jjd
qpoooekfjg vbzxx dsg forth a

Splice Junction Mutations

- GT/AG rule
  - AAGGTAAGT. .. . . //. .. . YYYYYYYYYYNCAGG
- Loss of splice site
  - intron incorporated in mRNA
- Creation of *novel* splice sites
  - >100 mutations
    - e.g., Hemoglobin E

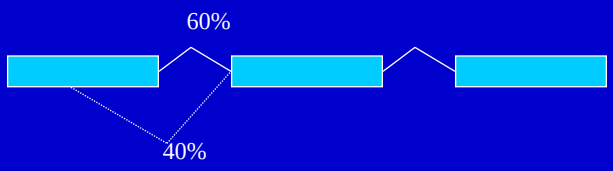
–Hemoglobin E

–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

Insertions

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

Frame-Shift Mutations

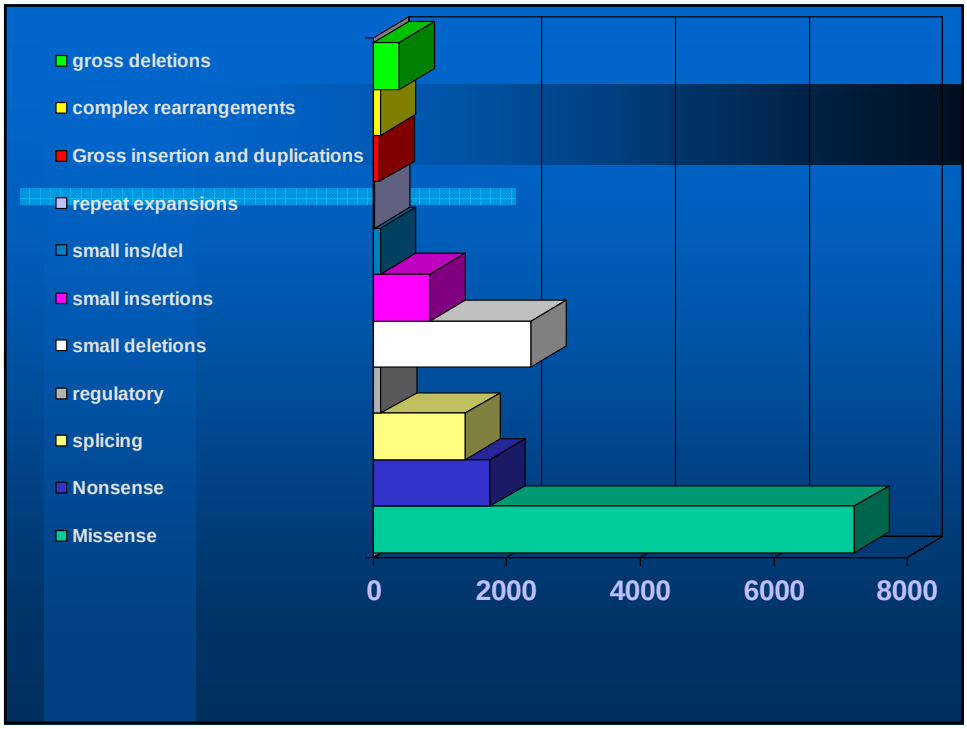
- Codon = 3 bp
- insertion/deletion not multiple of 3bp
  - Change of reading frame - entire protein altered.
  - e.g., Tay Sachs 4 bp insertion, BRCA1 185 delAG, BRCA2 6174delT, etc.
  - blood group O (1 bp deletion)

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**
    - **Friedrich's Ataxia (GAA)n intron**
  - **3'UTR**
    - **Myotonic dystrophy (CTG)n 3'UT**



"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.
  - Ethnicitiy
    - 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).**

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.**

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

Group	Incidence	Carrier freq.	%ΔF508	% other "common"	% group-specific	Sensitivity
Caucasian	1:3,300	1/29	70	13		80-90%
Hispanic	1/8-9000	1/46	46	11		57%
Ashkenazim	1:3,300	1/29	30	67		97%
Native Am.	1:1500 – 3970		0	25	69	94%
African Am.	1:15,300	1:60-65	48	4	23	75%
Asian Am.	1:32,100	1:90	30	0	0	30%

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-.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**

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**

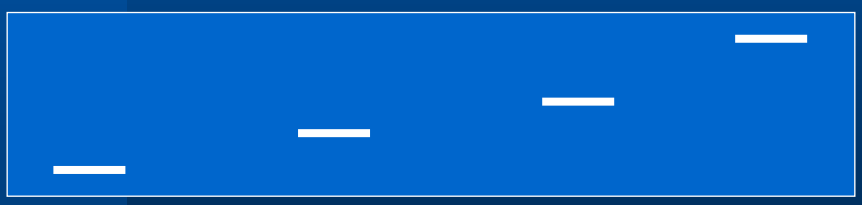
Expanded trinucleotide repeats

- **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.

Mutation Scanning Methods

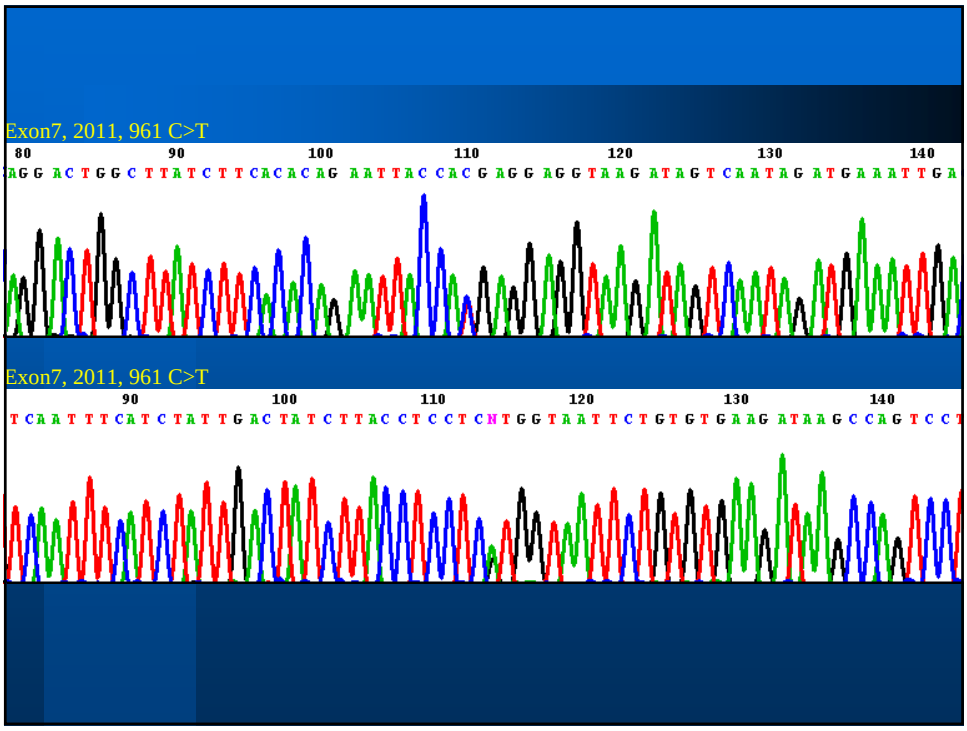
- Screening "physical properties"
  - Test for altered denaturation profile, or electrophoretic mobility
  - e.g., SSCP, DGGE, DHPLC, Cleavase fragment length polymorphisms, heteroduplex analysis, dideoxy fingerprinting.
  - Sensitivity varies for different genes/mutations
  - Need to use multiple conditions
  - *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
  - 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.

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

Modified from: BMJ: 322: 1054; April 28, 2001.

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].**