# Molecular Diagnosis

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

## What is a "Genetic Test"?

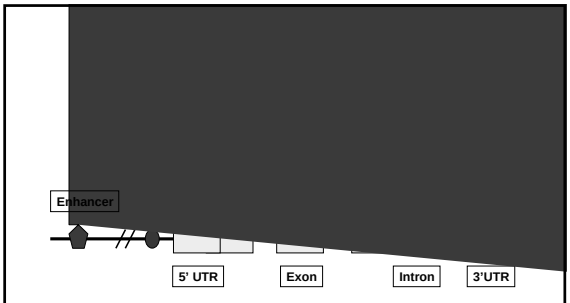
- Analysis of human
  - DNA, RNA, chromosomes, proteins, metabolites
- to detect heritable disease related
  - genotype, mutation, phenotype, or karyotype
- for *clinical purposes.*
- *"Molecular"* genetic test: DNA/RNA-based
  - Usu. PCR or related methodology, or Southern Blotting.

## 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

## Nuclear genome organization

- **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.




## Mitochondrial genome

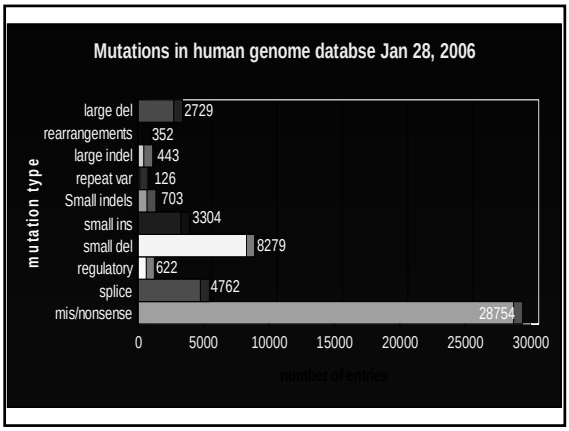
- 16,569bp, 44% GC
  - "H" strand – rich in G; "L" strand – rich in C.
- 37 genes – 28 encoded on "H", 9 on "L"
  - 22 tRNA, 2 rRNA
  - 13 polypeptide genes
    - 13 of > 80 subunits of respiratory complexes of oxidative phosphorylation system.
- Variable number per cell.
- "**Heteroplasmy**"

## Mitochondrial Genetic Code

| Codon | Nuclear Code | Mitochondrial code |
|---|---|---|
| AGA | Arg | Stop |
| AGG | Arg | Stop |
| UGA | Stop | Trp |
| AUA | Ile | Met |

## 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

| **Change** | **Example** |
|---|---|
| Entire gene deletion | α-thalassemia |
| Partial gene deletion | 60% of DMD |
| Insertions | LINE-1 insertion in F8 |
| Translocations | Women w/ DMD |
| Inversion | F8 inversion |
| Promoter mutation | β-globin -29 A>G |
| Promoter methylation | Many cancers |
| Poly-A site mutation | α-globin AATAAA>AATAGA |

## Loss of Function mutations

| Change | Example |
|---|---|
| Nonsense mediated RNA decay | Beta-globin Q39X |
| Splice donor loss | PAX3 451+1 G>T |
| Splice acceptor loss | PAX3 452-2 A>G |
| Δ Exonic splicing enhancer | SMN2 Exon7 change |
| Activate cryptic splice site | CFTR 3849 +10kb C>T |
| Frame-shift mutation | BRCA1 185delAG |
| Nonsense mutation | PAX3 Q254X |
| Missense mutation | HFE C282Y |

## 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 vs DNA

- 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.

## Tests for known mutations

- Many proprietary technologies.
- Each with its own advantages and pitfalls.
  - Pitfalls known to laboratory.
  - In New York State, report must indicate the limitations/pitfalls.
- Positive result – usually unambiguous.
- Negative result:
  - Significance varies by condition.

## 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

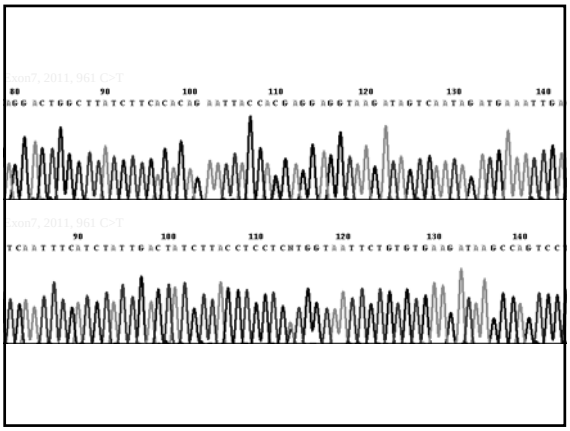
- physical properties of amplified gene segments
  - denaturation profile, electrophoretic mobility, etc.
    - SSCP (single strand conformation polymorphism)
    - DGGE (Denaturing gradient gel electrophoresis)
    - DHPLC (denaturing high performance liquid chromatography)
    - Cleavase fragment length 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
    - usu. multiple exons tested.
    - splice-site mutations may be missed, especially mutations deep in large exons.
  - RNA sequencing
    - need for cells w/c express gene
    - "nonsense mediated decay"
    - RNA more lablile



## 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.

## 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: BY POPULATION

| 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% |

## 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" = 00958
    - probability of affected child ~ 1 in 43,584

## Negative results, Screening

- Asian Couple:
  - Carrier rate = 0.011
    - probability of affected child ~ 1: 32,000
  - "Residual Risk" = .0077
    - probability of affected child ~ 67500

## Negative results: + family history

- 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

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

**Normal** **At risk for expansion** **Variable penetrance** **Affected**




## 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" missense 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.).

## 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/mortality of ds
  - 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/?cl=17&a=12].