Genetic testing using methods of molecular biology

- Molecular diagnosis of inherited diseases - 3500 genes with disease-causing mutations
- Prenatal diagnosis – trisomy detection, noninvasive prenatal testing
- Hematology – e.g. trombophilic mutations
- Molecular oncology – molecular diagnosis of leukemia, lymphoma, prediction of targeted therapy
- Detection and identification of microorganisms and viruses - hepatits B, C viruses, HPV,
- DNA-based tissue typing
Molecular basis of single-gene disorders - OMIM

14000 genes putative disease-association
3500 genes with disease-causing mutations

Molecular testing
- confirmation of the clinical diagnosis
- differential diagnosis
- presymptomatic diagnosis
- diagnosis in the family
- prognosis und severity

Methods
1. PCR and successive dideoxysequencing
2. Next-generation sequencing

Interpretation
1. Bioinformatic tools - BLAST, GenBank, commercial SW
2. Mutation databases – e.g. BIOBASE

Molecular diagnosis of inherited diseases

- Field of medical genetics, based on the analysis of molecular nature of monogenic diseases
- Patterns of inheritance are determined by examination of family histories

Pedigree is a diagram of the inheritance pattern of a phenotype of family members
- Autosomal-dominant
- Autosomal-recessive
- X-linked or sex-linked
Autosomal dominant disorders

1. Assume particular relevance because mutations in a single gene are sufficient to cause the disease.
2. Individuals are affected in successive generation; the disease does not occur in the offspring of unaffected individuals.
3. Males and females are affected in equal frequency.
4. The probability that an offspring will be affected is 50%.
5. Children with a normal phenotype do not transmit the disorder.
6. Due to the differences in penetrance or expressivity, the clinical manifestation of autosomal dominant disorders may be variable.

Characteristics of autosomal recessive inheritance

1. An autosomal recessive phenotype, if it appears in more than one member of a kindred, typically is seen only in the sibship of the proband, not in parents, offspring or other relatives.
2. For most autosomal recessive diseases, males and females are equally likely to be affected.
3. Parents of an affected child are asymptomatic carriers of mutant alleles.
4. The parents of an affected person may in some cases be consanguineous. This is especially likely if the gene responsible the condition is rare in the population.
5. The recurrence risk for each sib if the proband is 1 to 4.
Characteristics of X-linked inheritance

(Males have only one X chromosome; consequently, a daughter always inherits her father's chromosome in addition to one of her mother's two X chromosomes. A son inherits the Y chromosome from his father and one maternal X chromosome.)

1. The absence of father-to-son transmission
2. The fact that all daughter of an affected male are obligate carries of the mutant allele

X-linked recessive – affects mainly males;
affected males are usually born to unaffected parents; the mother is normally an asymptomatic carrier and may have affected male relatives

X-linked dominant – affects either sex, but more females than males;
Females are more mildly and more variable affected than males;
The child of an affected female, regardless of its sex, has 50% chance of being affected

Penetrance

• refers to the proportion of individuals with a mutant genotype that express the phenotype.
• If all carriers of a mutant express the phenotype, penetrance is complete, whereas it is said to be incomplete or reduced if some individuals do not have any features of the phenotype.
• Dominant conditions with incomplete penetrance are characterized by skipping of generations with unaffected carriers transmitting the mutant gene. For example, hypertrophic obstructive cardiomyopathy breast cancer caused by mutations in the BRCA 1 or BRCA 2 genes is a dominant disorder with clinical features in only a subset of patients who carry the mutation.
• Patients who have the mutation but no evidence of the disease can still transmit the disorder to subsequent generations.
• When describing penetrance, one has to specify age. For example, for disorders such as Huntington disease, which present late in life, the rate of penetrance is influenced by the age at which the clinical assessment is performed.
• cumulative cancer risks for mutation carriers BRCA 1 at age 70 years were as follows: breast cancer risk of 57% and BRCA 2 of 49%
Cystic fibrosis – Disease etiology and major phenotypic features

• CF is the most common fatal autosomal recessive disorder of Children in Caucasian populations of epithelial ion transport caused by mutation in the CF transmembrane conductance regulator gene (CFTR)
• Predominantly disease of Nothern Europeans (inc. 1:2500 Caucasina births; carrier frequency 1 in 25)
• Age of onset: neonatal to adulthood
• Progressive pulmonary disease
• Exocrine pancreatic insufficiency, elevated sweat chloride

Since the 1960s, CF has been one of the most publicly visible of all human genetic diseases.
A single mutation, a deletion of 3 bp, that results in the loss of a phenylalanine residue at position 508 in the predicted amino acid sequence.

This mutation accounts for 70% of all mutations at the locus in populations of Northern European descent.

The location of the cystic fibrosis gene on chromosome 7, with an enlargement showing the nature of the common 3-bp deletion that removes a phenylalanine from the polypeptide sequence.

**Six most common CFTR mutations in Caucasians**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Relative frequency in % (mut. class)</th>
</tr>
</thead>
<tbody>
<tr>
<td>deltaF508 – aminoacid deletion</td>
<td>66 (II)</td>
</tr>
<tr>
<td>G542X – stop codon</td>
<td>2,4 (I)</td>
</tr>
<tr>
<td>G551D – amino acid substitution</td>
<td>1,6 (III)</td>
</tr>
<tr>
<td>N1303K – aminoacid substitution</td>
<td>1,3 (II)</td>
</tr>
<tr>
<td>W1282X – stop kodon</td>
<td>1,2 (I)</td>
</tr>
<tr>
<td>R553X</td>
<td>0,7 (I)</td>
</tr>
</tbody>
</table>

*from: [http://www.genetests.org](http://www.genetests.org)*
Although the biochemical abnormalities associated with most CF mutations are not known, five general mechanisms of protein dysfunction have been described

### Classification of CFTR mutations

<table>
<thead>
<tr>
<th>Mutation Class</th>
<th>Effect of Mutation on CFTR Protein</th>
<th>Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Absent synthesis</td>
<td>Nonsense, frameshift, or splice-junction mutations</td>
</tr>
<tr>
<td>II</td>
<td>Block in protein processing</td>
<td>Missense mutations, amino acid deletions</td>
</tr>
<tr>
<td>III</td>
<td>Block in regulation of CFTR chloride channel</td>
<td>Missense mutations</td>
</tr>
<tr>
<td>IV, V</td>
<td>Altered conductance of CFTR chloride channel; reduced synthesis</td>
<td>Missense mutations, alternative splicing</td>
</tr>
</tbody>
</table>

### Pathophysiologic Classification of CFTR Mutations

<table>
<thead>
<tr>
<th>No synthesis</th>
<th>Block in processing</th>
<th>Block in regulation</th>
<th>Altered conductance</th>
<th>Reduced synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsense G542X</td>
<td>Missense</td>
<td>Missense G551D</td>
<td>Missense R117H</td>
<td>Missense A455E</td>
</tr>
<tr>
<td>Frameshift 394delTT</td>
<td>AA deletion</td>
<td>ΔF508</td>
<td></td>
<td>Alternative Splicing</td>
</tr>
<tr>
<td>Splice junction 1717-1G→A</td>
<td></td>
<td></td>
<td></td>
<td>3849+10kbC→T</td>
</tr>
</tbody>
</table>
Molecular diagnosis of CF – direct gene analysis and identification of disease-causing mutations

1. Sequencing of the complete gene
2. Searching for reported mutations

Reported mutations

PCR product scanning for mutations – distinguish homozygous and heterozygous

Autosomal dominant hereditary cancer syndrome

Figure 22-24 The genetic mechanisms underlying retinoblastoma. In the hereditary form, all cells in the body lack one of the normal two functional copies of the RB tumor suppressor gene, and tumors occur where the remaining copy is lost or inactivated by a somatic mutation. In the nonhereditary form, all cells initially contain two functional copies of the gene, and the tumor arises because both copies are lost or inactivated through the coincidence of two somatic mutations in one line of cells.
Using the known location of chromosomal deletion associated with retinoblastoma, it was possible to clone and sequence the gene whose loss appears to be critical for development of the cancer Rb gene. In those who suffer from hereditary form of disease, a deletion or loss-of-function occurs in one copy of the Rb gene in every cell of the body. Thus, cell are predisposed to becoming cancerous, but are not actually cancerous so long as they retain one good copy of the gene. The retinal cell that become cancerous are defective in both copies of Rb because a somatic mutation has occurred, in addition to the original inherited mutation, and has eliminated the remaining good copy. In patients with non hereditary retinoblastoma, the noncancerous cell show no defect in either copy of Rb gene, while cancerous cells are defective in both copies. These nonhereditary retinoblastoma are very rare, because their require coincidence of two somatic mutations in a single retinal cell linage.

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**Rare familial cancers caused by TS gene mutations:**

**Hereditary colon cancer and microsatellite instability.**

- **Hereditary nonpolyposis colon cancer** (HNPCC; OMIM 120435, 120436) is autosomal dominant and highly penetrant, but unlike FAP there is no preceding phase of polyposis. HNPCC genes were mapped to two locations, 2p15-22 and 3p21.3. The functions of the mismatch repair genes can be disrupted by missense mutations, truncating mutations, splice site mutations, large deletions or genomic rearrangements.

- **Familial adenomatous polyposis** (FAP or APC; OMIM 175100) is an autosomal dominant condition in which the colon is carpeted with hundreds or thousand of polyps. The polyps (adenomas) are not malignant, but if left in place, one or more of them is virtually certain to evolve into invasive carcinoma. The cause in an inherited mutation in the APC tumor suppressor gene – mutations almost always cause a premature truncation of the APC protein, usually through single amino acid substitutions or frameshifts. While mutations have been found scattered throughout the gene, they are predominantly located in the 5’ end of the gene.

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**Molecular diagnostics of the mutations in hereditary cancer syndromes**

1. **DNA isolation from peripheral blood**
2. **PCR of exons incl. ex./intr. boundaries**
3. **Premutational screening**
4. **Direct DNA sequencing**
Genetic testing

Test an affected family member first - after genetic counseling and informed consent

Molecular gene testing can confirm suspected diagnosis

Family members of a person with the known mutations can have mutation-specific testing

Strategies for genetic testing

1. Testing on known mutations – detection of presence/absence of specific mutations, e.g. sickle cell diseases – specific design of PCR

2. Testing of unknown mutations testing for presence of an unspecific change by comparing the PCR product of the wild-type control with the patient’s PCR product – different mutation screening strategies and confirmation by direct DNA sequencing

3. Next-generation sequencing
Methods for detection of unknown mutations after PCR

- Screening – detection of conformational changes within DNA - Finding heterozygotes among homozygotes within particular PCR product

Heteroduplex analysis (HDA)

- Confirmation of mutation
  Direct DNA sequencing

Heteroduplex analysis - heteroduplexes have abnormal mobility on nondenaturing PAGE

DNA of putative heteroallelic – heterozygous sample that contains wildtype and mutant is multiplied by PCR

Most mutations occur in heterozygous form (even with autosomal recessive conditions).
Metods based on detecting mismatches or heteroduplexes

Heteroduplexes can be formed simply by heating the heterozygous test PCR product to denature it, and then cooling slowly.

Observed Combination of 4 Duplexes

- C
- A
- T
- G

Heteroduplexes have abnormal mobility on nondenaturing PAGE

Heteroduplex analysis (HDA)

DGGE and dHPLC – the mobility of a fragment changes markedly when it denatures. These methods require tailoring to the particular DNA sequence under test, and are so best suited to routine analysis of a given fragment in many samples.
Genetic testing of monogenic diseases

- **Test an affected family member first!**
  - After genetic counseling and informed consent

- Gene testing confirm a suspected diagnosis
- Family members of a person with identified disease-causing mutation can have mutation specific testing

Pathogenic potential of repeated sequences – dynamic mutations

- Expansion of unstable trinucleotide STRs (microsatellite DNA)
  **Modest (CAG)n expansions resulting in polyglutamine tracts.** — stable are 10-30 repeats; unstable pathogenic alleles often have in the range 20,000 repeats – function of polyG tracts causes the protein to aggregate within certain cells and kill them; gain of function — Huntington disease
  **Very large noncoding repeat expansions** — expansions inhibit expression of closely neighboring genes, causing loss of function. Stable, nonpathogenic allele have 5-50 repeats; unstable pathogenic alleles have several hundreds or thousands of copies; very large expansions affect chromosome structure, causing **fragile sites** — fragile X syndrome
Effects of expanded trinucleotide repeats

• Very large expansions in noncoding regions
  Loss of function (Fragile X syndrome (OMIM: 309550), Friedreich ataxia (OMIM: 229300),
• Modest expanding coding regions
  (poly-Glu regions)
  Gain of function (Huntington disease (OMIM: 143100))

Fragile X Syndrome type A (FRAXA)- Martin Bell syndrome

Is the most common heritable form of moderate mental retardation and is second form only to Down syndrome among all causes of moderate mental retardation in males with prevalence 1:4000-6000

• The name „fragile X“ refers to a cytogenetic marker on the X chromosome at Xq27.3 a „fragile site“ in which the chromatin fails to condense properly during mitosis
• Clinical features – mental retardation (IQ 20-60), long face with large madibulla, large ears, hyperactivity
Fragile X syndrome

The disorder is caused by a dynamic mutation, a massive expansion of a triplet repeat CGG, located in the 5’ untranslated region of the first exon of a gene called FMR1. The normal number of repeats is up to 50, whereas many several thousand repeats are found in patients with the full fragile X syndrome.

Methylation

HD huntingtin

- Gene IT-15 coding for protein huntingtin
- Exon 1 consists high polymorphic CAG
- Modest expansion CAG encodes polyglutamine tract in the gene product inducing death of neuron cells.
- Gain-of function mutation
- Molecular diagnostics: PCR on PAGE gel or fragmentation analysis via CE - determination of the number of the CAG repeats
- Less than 27 – normal phenotype
- More than 40 - mutation
- 27-34 copies – non risk for the carrier, can expand in the next generation.
- 35-39 borderline – penetrance not complete
A fragment of the gene containing the (CAG)\textit{n} repeats has been amplified by PCR and run out on PAGE. Bands are revealed by silver staining. The scale shows number of repeats.

Lanes 1, 2, 6, and 10 are from unaffected people,

Lanes 3, 4, 5, 7, and 8 are from affected people. Lane 5 is juvenile onset case; her father (lane 4) had 45 repeats but she has 86.

Lane 9 is an affected fetus, diagnosed prenatally.

Strachan and Read, 2004

<table>
<thead>
<tr>
<th>Genetic Disorder</th>
<th>Inheritance</th>
<th>Genes</th>
<th>Interventions</th>
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</thead>
<tbody>
<tr>
<td>Oncologic</td>
<td></td>
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<tr>
<td>Hereditary nonpolyposis colon cancer</td>
<td>AD</td>
<td>MSH2, MLH1, MSH6, PMS1, PMS2, 70BP2</td>
<td>Early endoscopic screening</td>
</tr>
<tr>
<td>Familial adenomatous polyposis</td>
<td>AD</td>
<td>APC</td>
<td>Early endoscopic screening</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Nonsteroidal anti-inflammatory drugs</td>
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<td></td>
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<td></td>
<td>Colectomy</td>
</tr>
<tr>
<td>Familial breast and ovarian cancer</td>
<td>AD</td>
<td>BRCA1, BRCA2</td>
<td>Estrogen receptor antagonists</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Early screening by exam and mammography</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Consideration of prophylactic surgery</td>
</tr>
<tr>
<td>Familial melanoma</td>
<td>AD</td>
<td>CDKN2A</td>
<td>Avoidance of UV light</td>
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<td></td>
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<td>Screening and biopsies</td>
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<tr>
<td>Basal cell nevus syndrome</td>
<td>AD</td>
<td>PTH</td>
<td>Avoidance of UV light</td>
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### Methods used for detection of mutations I

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Type of Mutation Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Commonly Used Techniques</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytogenetic analysis</td>
<td>Unique visual appearance of various chromosomes</td>
<td>Numerical or structural abnormalities in chromosomes</td>
</tr>
<tr>
<td>Fluorescence in situ hybridization (FISH)</td>
<td>Hybridization to chromosomes with fluorescently labeled probes</td>
<td>Numerical or structural abnormalities in chromosomes</td>
</tr>
<tr>
<td>Southern blot</td>
<td>Hybridization with genomic probe or cDNA probe after digestion of high molecular DNA</td>
<td>Large deletion, insertion, rearrangement, expansions of triplet repeat, amplification</td>
</tr>
<tr>
<td>Polymerase chain reaction (PCR)</td>
<td>Amplification of DNA segment</td>
<td>Expansion of triplet repeats, variable number of tandem repeats (VNTR), gene rearrangements, translocations, prepare DNA for other mutation methods</td>
</tr>
<tr>
<td>Reverse transcription PCR (RT-PCR)</td>
<td>Reverse transcription, amplification of DNA segment + absence or reduction of mRNA transcription</td>
<td>Analysis expressed mRNA (cDNA) sequence; detect loss of expression</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>Direct sequencing of PCR products</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>Restriction fragment polymorphism (RFLP)</td>
<td>Detection of altered restriction pattern of genomic DNA (Southern blot or PCR products)</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
</tbody>
</table>

### Methods used for detection of mutations II

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Type of Mutation Detected</th>
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</thead>
<tbody>
<tr>
<td><strong>Other Techniques</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single-strand conformational polymorphism (SSCP)</td>
<td>PCR of DNA segment. Mutations result in conformational change and altered mobility</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>Denaturing gradient gel electrophoresis (DGGE)</td>
<td>PCR of DNA segment. Mutations result in conformational change and altered mobility</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>RFLA digestion</td>
<td>Cleavage of mismatch between mutated and wild-type sequence</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>Oligonucleotide specific hybridization (OSH)</td>
<td>Hybridization of PCR products to wild-type or mutated oligonucleotides immobilized on chips or slides</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>Microarrays</td>
<td>Hybridization of PCR products to wild-type or mutated oligonucleotides</td>
<td>Point mutations, small deletions and insertions</td>
</tr>
<tr>
<td>Protein truncation test (PTT)</td>
<td>Transcription/translation of cDNA isolated from tissue sample</td>
<td>Mutations leading to premature truncations</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Clonal amplification of single DNA fragments on microarrays followed by massive parallel sequencing</td>
<td>Sequencing of whole genomes of microorganisms, resequencing of amplicons</td>
</tr>
<tr>
<td>Multiplex ligation-dependent probe amplification (MLPA)</td>
<td>Quantification of PCR-generated amplicons reflecting the number of copies of a specific DNA sequence</td>
<td>Copy number variations</td>
</tr>
</tbody>
</table>
Interpretation of mutations for research and diagnostics

- Type of inheritance and / or disease – test strategy
- Type of mutations - gain of function/loss of function mutation plus typical sequence changes (point mutations, missense mutations, small deletions, insertions, frameshift mutations, dynamic mutations) - choice of method; describing mutation
- Searching in general databases for mutations
- Searching in specified databases for each disease

<table>
<thead>
<tr>
<th>Site</th>
<th>URL</th>
<th>Comment</th>
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<tbody>
<tr>
<td>National Center for Biotechnology Information (NCBI)</td>
<td><a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a></td>
<td>Molecular biology information, public databases, computational biology. Software for analyzing genome data. Extensive links to other databases, genome resources, and tutorials</td>
</tr>
<tr>
<td>National Human Genome Research Institute</td>
<td><a href="http://www.genome.gov/">http://www.genome.gov/</a></td>
<td>Web links providing information about the human genome sequence, genomes of other organisms, and genomic research</td>
</tr>
<tr>
<td>Ensembl Genome browser</td>
<td><a href="http://www.ensembl.org/">http://www.ensembl.org/</a></td>
<td>Maps and sequence information of eukaryotic genomes</td>
</tr>
<tr>
<td>Office of Biotechnology Activities National Institutes of Health</td>
<td>www4.od.nih.gov/oba/</td>
<td>Information about recombinant DNA and gene transfer. Medical, ethical, legal, and social issues raised by genetic testing</td>
</tr>
<tr>
<td>Database Name</td>
<td>Website</td>
<td>Description</td>
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<tr>
<td>---------------------------------------------------</td>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>American College of Medical Genetics</td>
<td><a href="http://www.acmg.net/">http://www.acmg.net/</a></td>
<td>Extensive links to other databases relevant for the diagnosis, treatment, and prevention of genetic disease</td>
</tr>
<tr>
<td>GenLink</td>
<td><a href="http://www.genlink.wustl.edu">http://www.genlink.wustl.edu</a></td>
<td>Multimedia database resource for human genetics and telomere research</td>
</tr>
<tr>
<td>Genomes Online Database (GOLD)</td>
<td><a href="http://www.genomeonline.org/">http://www.genomeonline.org/</a></td>
<td>Information on published and unpublished genomes</td>
</tr>
<tr>
<td>HUGO Gene Nomenclature</td>
<td><a href="http://www.gene.ucl.ac.uk/nomenclature">http://www.gene.ucl.ac.uk/nomenclature</a></td>
<td>Gene names and symbols</td>
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</table>