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19. MOLECULAR DIAGNOSIS OF NEUROMUSCULAR DISEASE

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Recent progress in molecular genetics has greatly expanded our knowledge of the molecular basis of many inherited disorders, particularly neurological diseases.

There are a large number of mutated genes that cause neurological dysfunction, and in many cases mutations have been identified. This increasing wealth of knowledge in neurogenetics is valuable in classification and reclassification of a number of heterogeneous diseases and provides novel diagnostic possibilities. Molecular approaches allow the characterization of pathological gene mutations and gene products, providing new insight into the molecular pathogenesis of inherited neurological disorders. Molecular diagnosis is important because it provides valuable information for affected individuals and for entire family members. Also, the identification of disease genes allows presymptomatic and prenatal diagnosis of neurological diseases. In the laboratory, the limitation of molecular diagnosis depends on the degree of genetic complexity of disorders, because some diseases are caused by a specific mutation in a single gene and routine molecular diagnosis can be provided by a simple DNA assay, but in other diseases many different mutations can be found and DNA assay is more complex. Genotyping is useful for many neurologic diseases, but this review is designated to describe the possibility of molecular diagnosis for severe and most frequent diseases such as Duchene/Becker muscular dystrophy, spinal muscle atrophy and Huntington's disease.

19.1 Principles of molecular diagnosis

19.1.1 Direct molecular diagnosis based on the analysis of gene mutations

Direct molecular diagnosis can be performed if the gene causing a neurological disease is known. Only DNA from an affected individual is required. Usually, DNA is extracted from peripheral blood leukocytes or amniotic cells and exonic sequences, which are known to have mutations in the particular disease, will be amplified by use of the polymerase chain reaction (PCR). Depending of the type of mutation, it will be detected either directly by gel electrophoresis or using hybridization. If a gene is large and mutations are present throughout the entire gene, direct sequence analysis is offered for portions of a gene, where mutations may be clustered.

19.1.2 Indirect molecular diagnosis based on the analysis of DNA polymorphisms

Knowledge of the chromosomal position of a disease gene allows molecular support for the diagnosis, even if the disease gene is unknown or if analysis is not feasible. Indirect molecular diagnosis is limited to risk determination for an individual in whose family an inherited neurological disease has already been diagnosed. The method is based on the analysis of DNA polymorphism closely linked to the disease gene. Determination of the polymorphic alleles in healthy and affected family members allows the identification of the disease-causing genes.

19.2 Duchenne / Becker Muscular Dystrophy

Duchenne and Becker muscular dystrophy (D/BMD) are progressive lethal disorders caused by mutations in the dystrophin gene. Dystrophin is a membrane-associated protein complex in skeletal muscle fibre and connective tissue. Due to the X-linked nature of D/BMD males carrying the mutated gene are affected, while females become carriers of the disease. Diagnosis of patients is usually definitively based on clinical, pathological, biochemical and molecular findings. The dystrophin gene involved in D/BMD codes muscle-specific protein named dystrophin as well as several tissue-specific isoforms. Identification of causative mutations in the dystrophin gene can provide an accurate diagnosis for the affected individual, determination of carrier status, and it provides options for prenatal diagnosis. Accurate diagnosis of these disorders becomes crucial for counselling and managing patients. The dystrophin gene is about 2.4 Mb in size, consisting of 79 exons, and is localized at Xp21. Most mutations in the gene are in two hotspots located at the proximal and central regions of the gene, called the "deletional hotspots". About 65%-70% of patients show intragenic deletions of one or several exons of the gene that can be detected by multiplex PCR or Southern blotting. For carrier status, quantitative and real time PCR are usually carried out. Other techniques, such as FISH (Fluorescent in situ hybridization), can be used for carrier detection, as well as single-strand conformation polymorphism (SSCP), reverse transcription RT-PCR for point mutation detection, but are not feasible for routine diagnostic purposes. In addition, Western blot of dystrophin from dystrophinopathies DMD/BMD can be carried out to detect the size and amount of protein.

19.2.1 Multiplex-PCR

Rapid detection of deletions by PCR allows proper DMD/BMD diagnosis in males. Specific DNA regions are amplified in multiplex reaction according to Chamberlain and Beggs. Laboratories usually use 25 pairs of primers for detection of about 98% of deletions. The deletions can be identified from the pattern of bands as visualized on ethidium-bromide stained agarose gels. Once the deletion has been detected, it becomes a marker for the family and prenatal diagnosis can easily be provided.

This method cannot be used for determination of carrier status in female subjects because of heterozygosity.

19.2.2 Southern blotting

Southern blotting is the method for detection of deletions or duplications of one or more exons of the gene. In this assay, the DNA is digested by restriction enzymes that recognize a specific site. The restriction fragments are subjected to electrophoresis and hybridized to complementary cDNA probes. The labeling of probes by radioactive nucleotides (P32) allows detection of deletions or duplications by autoradiography. 5-10% affected males have duplications of exons.

19.2.3 Real time PCR

This is automated PCR quantitation for detection of carrier status in females. In real time assay, the progress of PCR can be monitored and reaction can be terminated at the desired point.

19.2.4 Linkage analysis

Linkage analysis that usually employs intragenic dinucleotide (CA) repeat of dystrophin gene for carrier detection. Intragenic STRs (3' DYS MS, 5' DYS, STR 44, STR 45, STR49, STR50) markers of dystrophin gene provide carrier status and can be used for prenatal diagnosis. This molecular diagnosis requires the sample of proband and family members for haplotyping (Fig.1).

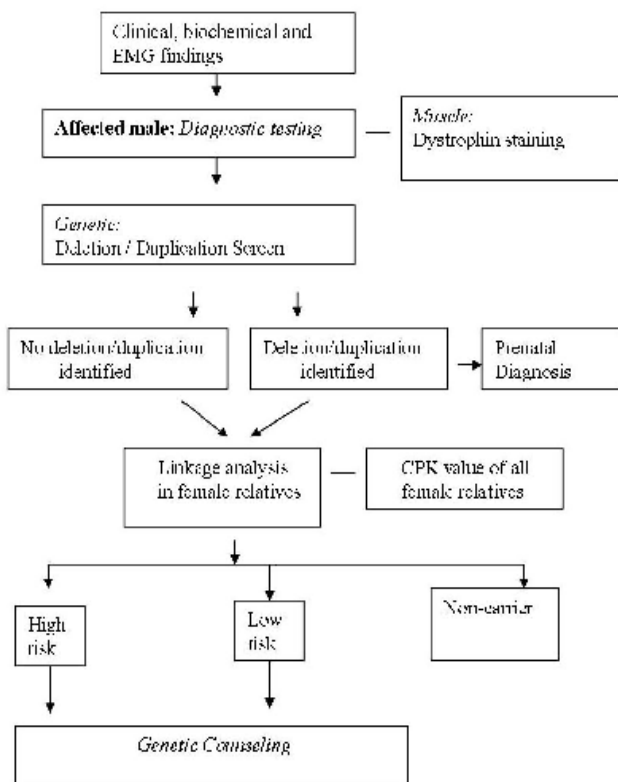


Figure 1. Approach to molecular diagnosis for D/BMD.

19.2.5 Spinal muscle atrophy

Motor neurone diseases are caused by selective motor neurone degeneration, presenting with progressive muscle weakness and atrophy. Spinal muscular atrophy (SMA) is the most common motor neurone disease in children with the incidence of 1/6,000 - 1/

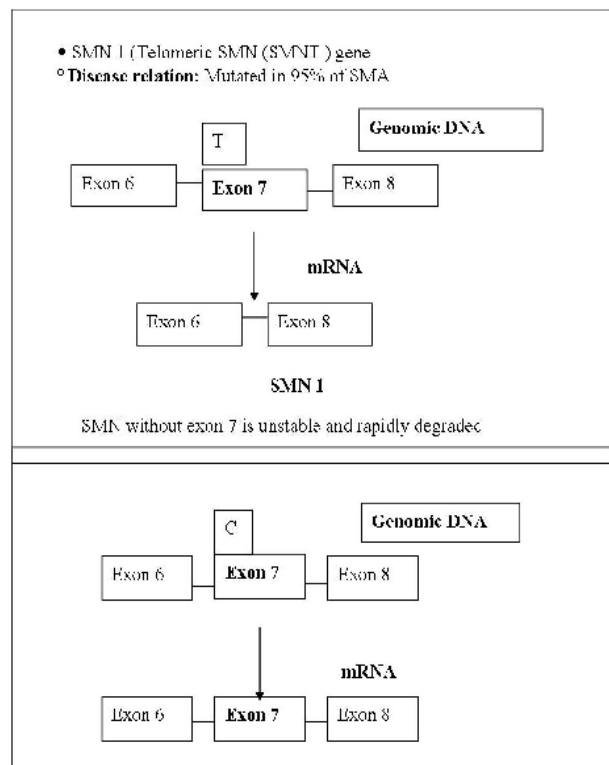
10,000. SMA was described independently by Werdnig and Hoffmann in 1891. Werdnig described the condition as "neurogenic dystrophy" and Hoffmann established the spinal nature of the disease. The International Consortium has distinguished SMA variants and classical SMA (types I, II, and III). Type I (Werdnig-Hoffman disease) is the most acute and severe form, with onset before the age of 6 months and death usually before the age of 2 years, type II (intermediate chronic form) has onset before the age of 2 years, type III (Kugelberg-Welander disease) is a mild form, with onset after the age of 18 months, while type IV is characterized by late onset, slow progression and proximal weakness. The genes responsible for SMA are: survival motor neurone gene (SMN), neuronal apoptosis inhibitory protein gene (NAIP), basal transcription factor subunit (Btf2-p44), and microsatellite marker (C212/H4F5). SMN has telomeric (SMN1 or SMNT) and centromeric (SMN2 OR SMNC) copy, which differ in only five nucleotides at their 3' end (Fig.2).



Figure 2. SMA related or neighboring genes on chromosome 5q

Large inverted duplication has telomeric and centromeric copies. Telomeric genes are functional genes, while centromeric genes represent dysfunctional copies. In classical SMA, the majority of patients (95%) show homozygous deletion of the telomeric SMN gene (SMN1) on chromosome 5q11.2-q13.3. Homozygous SMN2 deletion is present in 5% of normal

Figure 3. SMA: Molecular diagnosis



The molecular diagnosis of SMN gene deletions can be carried out by PCR and restriction fragment length polymorphism (RFLP). The telomeric and centromeric copies can be identified by selective restriction enzyme digestion of DNA (Fig.3). For exon 7, PCR product is digested with DraI restriction enzyme and visualized with ethidium bromide on agarose gel electrophoresis. Similar for

exon 8, the restriction enzyme is DdeI. Detection of point mutations requires specialized techniques like single SSCP. Absence of exon 7 of SMN1 gene has become a diagnostic tool for confirmation of the disease and prenatal diagnosis. Identification of SMA carriers is possible by quantitative PCR-based assays for the determination of SMN1 copy number. Preimplantation genetic diagnosis in families at risk has also been reported.

Molecular diagnosis of SMA in Croatia has been performed on 265 patients, including 20 prenatal tests. Deletion of exons 7/8/5 of telomeric SMN and NAIP genes was established in all SMA type I patients, in 59% patients with type II and in 23% patients with type III. Also, homozygous centromeric deletion SMN2 was identified in one atypical SMA patient.

Huntington's disease

Huntington's disease (HD, Huntington Chorea), a progressive disorder of motor, cognitive, and psychiatric disturbance usually occur at middle age. The nature of the genetic defects is an unstable expanded CAG repeat within the coding region of HD gene on chromosome 4p16. Gene product is protein Huntington that can be normal or abnormal. The CAG in the gene is translated into polyglutamine tracts that may be toxic to cells. The prevalence of HD is between 3 and 7 per 100,000 in European population.

Normal

10 - 26 repeats

CAGCAGCAGCAGCAG →
GTCGTCGTCGTC

Altered

36 - 121 repeats

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG →
GTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTC

Figure 4. DNA (CAG) repeat sequences of HD gene

A single diagnostic procedure can be performed to confirm the presence of a mutation associated with HD. The number of CAG repeats ranges from 10 to 26 in normal alleles. In patients with HD, the CAG repeat number ranges from 36 to 121 (pathologic allelic variants). Alleles that are the size of 27-35 CAG repeats are considered intermediate alleles; a person with an allele in the intermediate range may be at risk for having a child with an allele in the abnormal range, but the person is not at risk to develop symptoms of HD. An allele in the range of 36-41 CAG repeats is associated with "reduced penetrance" for symptomatic HD; an individual with an allele in the range of reduced penetrance may or may not develop symptoms of HD in lifetime (Fig. 4). The diagnosis of HD is based on positive family history, characteristic clinical findings and molecular diagnosis that reveals an expansion of the CAG repeat by PCR-assay. DNA-based testing is 98.8% sensitive and is widely available. HD is an autosomal dominant disease.

The offspring of an individual with a mutant allele have a 50% chance of inheriting the disease-causing allele. Molecular diagnosis includes: confirmatory and diagnostic testing, predictive testing, and prenatal testing. Although infrequently requested, prenatal diagnosis may be performed when only one parent carries the HD gene and the couple wants to determine the carrier status of the fetus. Prenatal testing for fetus at 25% risk can be performed using linkage analysis. Anticipation occurs more commonly in paternal transmission of the mutated allele. The phenomenon of anticipation arises from instability of CAG repeat during

spermatogenesis. Most often children with juvenile-onset disease have inherited the expanded allele from their fathers, and usually have expansions above 60 CAG repeats. The identification of HD genetic defect is possible by direct DNA test through PCR amplification of CAG repeat. The genotype-phenotype model shows a significant inverse correlation between the number of CAG repeats and the age, and homozygotes are no more severely affected than heterozygotes.

Neurologic diseases are an important group of inherited disorders with genetic heterogeneity. Molecular diagnosis allows carrier detection, postnatal and prenatal diagnosis in the affected families. Therapeutic modalities according to genotype are still underway.

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