PCR IN MEDICINE III

The Congress centre of the Institute of Clinical and Experimental Medicine in Prague was the site of the seminar PCR in Medicine III that took place on 21st November 2001. The seminar was organised by Sigma-Aldrich in collaboration with Institute of Inherited Metabolic Disorders of the 1st Medical Faculty, Charles University, and General Faculty Hospital in Prague. The programme of the seminar was devoted to the exploitation molecular biology in medical research and diagnostics. 10 presented lectures brought reliable and illustrating overview of recent techniques used in this special field of medicine to about 140 participants that took part in the seminar.

ABSTRACTS

AUTOREFERÁTY PŘEDNÁŠEK

K. Klepárník (Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Brno): **Potentiality of capillary electrophoresis in DNA diagnostics**.

The growing demands for rapid and large-scale DNA sequencing and mutational analysis have stimulated an intensive research in the field of high-throughput analytical methods. As a result, the development of capillary electrophoresis in the past decade brought innovations that became the essential prerequisite of the sequencing the total DNA of the human genome in only nine months. Not only DNA sequencing, but also analyses of DNA fragments length and conformation polymorphism (known as RFLP, AFLP or SSCP techniques, respectively), polymorphism of short tandem repeats, heteroduplex (HDA) and denaturing analyses (CDCE) have been adopted for capillary format. An optimized methodology of electrophoresis in short capillaries with laser-induced fluorescence detection results in a reduction of analysis time by a factor of 100 when compared to the conventional slab gel electrophoresis. The technique is especially advantageous in clinical diagnostics and genetic population screening where a fast analytical instrumentation amenable to automation is of paramount importance.

Reliable and high-throughput analyses of mutations causing serious hereditary metabolic disorders, analyses of short tandem repeat polymorphism and fast DNA diagnostic sequencing will be presented as examples. Denaturing capillary electrophoresis provides much faster and more selective separations of DNA fragments than electrophoresis under native conditions. The denaturing effectiveness of commonly used organic denaturants, however, is not satisfactory toward guanine and cytosine rich DNA fragments. To overcome this shortcoming, authors have developed a highly alkaline electrolyte system with remarkable separation properties. Thus, the analyses of a microsatellite repeat polymorphism in Endothelin 1 gene under denaturing conditions in short capillaries were accomplished in 42 seconds.

The implementation of fully automated capillary array electrophoresis with comprehensive fraction collection in the fields of gene expression analysis and mutation discovery will be shown. In this preparative technique, all fractions migrating from capillaries are collected and then available for amplification, sequencing or any further analysis. The technology was tailored to a fast gene expression analysis, genome-wide DNA profiling applications and mutation detection in many pooled samples.

M. Matějčková, M. Broučková (Department of Biology and Medical Genetics, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague): **Capillary electrophoresis in prenatal and postnatal diagnosis of cystic fibrosis and the most common aneupoidies**.

Automated fluorescent scanning detection of DNA fragments allows measurement of particular lengths of DNA fragments and their relative quantity based on their normalized mobilities. Capillary electrophoresis successfully replaces classical methods (agarose electrophoresis, PAGE electrophoresis) in cases of small length differences between mutated and normal fragments. An example is the case of F508del CFTR gene mutation in the Czech population. Such an approach is useful also in cases when it is for some reason difficult to detect PCR products or optimise the PCR reaction. Usage of fluorescent-labelled primers is very convenient for multiplex analysis, which we successfully use in prenatal and postnatal diagnostics. For urgent detection of 31 mutations in CFTR gene from microquantity of cells (33 ng of DNA) we use OLA kit (Oligonucleotide Ligation Assay – Applied Biosystems).

Authors use capillary electrophoresis for quantitative analysis of PCR products for prenatal and postnatal detection of aneuploidies of chromosomes 13,18, 21, X and Y. QFPCR (Quantitative Fluorescent Polymerase Chain Reaction) is rapid method for the detection of common numerical chromosome disorders and it plays an important role in prenatal diagnosis for women at high risk for fetal aneuploidy. This method uses specific, highly polymorph short tandem repeats (STR) markers located on the tested chromosomes.

R. Dobrovolný, L. Dvořáková, M. Hřebíček, J. Ledvinová, M. Elleder (Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine and General Faculty Hospital, Charles University, Prague): **DNA diagnostics of gonosomal recessive disorders: Mutation and X-inactivation analyses in patients with Fabry disease**.

Gonosomal recessive disorders belong to one of the five types of the basic Mendelian inheritance patterns. In this kind of inheritance, a gene linked to particular disease is localized on X sex chromosome and heterozygotes carrying at least one copy of wild type allele are not affected by the disease. On the other hand, it is important to keep in mind that due to X-inactivation in many heterozygous females some symptoms of a disease are manifested. As a model of gonosomal recessive disorder can serve Fabry disease.

Fabry disease, an X-linked inborn error of glycosphingolipid catabolism, results from the deficient activity of the lysosomal α-galactosidase A (E.C. 3.2.1.22). Affected individuals accumulate neutral glycosphingolipids with terminal α-linked galactosyl moieties particularly in the lysosomes of blood vessels, heart, and kidney. Diagnosis of Fabry disease is routinely done by the enzyme activity assay with artificial fluorogenic substrate (4-methyl-α–D-galactopyranosid). However, there is a risk of misdiagnosis of heterozygous carriers because their -galactosidase activities range from deficient to normal values. This problem could be reliably overcome by DNA diagnosis. DNA diagnosis is carried out in several subsequent steps:

Mutation identification in the family: Mutation can be determined from genomic DNA (gDNA) or from complementary DNA (cDNA). gDNA has the advantage of higher stability in comparison with cDNA (RNA). On the contrary, if gDNA is used it is necessary to sequence more fragments because coding regions of DNA in genome are interrupted by noncoding introns. In addition, there could be problems with interpretation of non-exonal sequence variations. In each case, the region of interest is amplified by PCR and PCR products are directly sequenced.

Mutation verification, molecular method for heterozygous detection: As soon as the mutation is identified, we design the rapid method for its detection. Among most widespread are RFLP or ARMS methods. These methods can be then used for reliable detection of heterozygous females or for prenatal diagnosis in the proband's family. To unravel the impact of X-inactivation status on clinical manifestation of Fabry disease in heterozygote females the authors optimized method for determination of X-inactivation pattern in several tissues. The method uses differences in methylation between active and inactivated X-chromosomes.

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M. Macek Jr. (Department of Biology and Medical Genetics – Centre for Cystic Fibrosis, 2nd Faculty of Medicine, Charles University, Prague): **Denaturing gradient gel electrophoresis (DGGE) in mutation scanning**.

Denaturing gradient gel electrophoresis (DGGE) is a generic designation for a family of related mutation scanning methods (e.g. CDGE, TGGE, TDGGE, CDCE, TTGE, TSGE, dgDGGE, gDGGE) that are based on the reduction of mobility in denaturing gel matrices due to gradual, cooperative, melting of distinct DNA domains within the analysed fragment. Unlike in DGGE the behaviour of a fragment in the gel can be predicted a priori with 100% accuracy. This is achieved by using computer programs (e.g. Melt94) that predict the melting profile (”meltmap”) of a given fragment. Gradually increasing denaturing conditions (chemicals – urea and formamide and/or temperature) during the course of electrophoresis physically separate wt from mutant strands. Formation of heteroduplexes in later phases of PCR considerably aids the identification of mutants. Resulting DGGE band patterns are highly mutation specific, allowing easy identification of previously identified alleles, even without the necessity of their sequencing. Similar to SSCP this technique is non-destructive and allows for isolation of mutant bands from complex DGGE patterns (which is particularly useful in the case of somatic mutations that usually form a minority in analysed tumour-biopsy DNA). The sensitivity and specificity of DNA is close to 100%. Although DGGE is difficult to set up, once optimised it provides an excellent system for identification of all mutations in a gene of interest, being thus optimal for molecular diagnostics settings. DGGE still has its justification in the ”high-tech sequencing era” by providing a much needed, economical, pre-scanning option for the majority of diagnostic and research laboratories.

M. Janošík[1]*, J. Oliveriusová*[2]*, B. Janošíková*[1]*, J. P. Kraus*[2] *and V. Kožich*[1] ([1]Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University Prague, Czech Republic; [2]Health Sciences Centre, University of Colorado, Denver, Co, USA): **Molecular mechanisms of mutations at the RNA and protein levels: nonsense mediated decay a protein aggregation**.

Homocystinuria due to cystathionine β-synthase (CBS) deficiency is the most common disorder of sulphur amino acid metabolism and it is inherited as an autosomal recessive trait. The disease is characterized by a total loss of CBS activity and by accumulation of homocysteine in body fluids and tissues. Clinical manifestation includes dislocation of the lenses, numerous skeletal abnormalities, mental retardation and thromboembolic events. So far, more than 114 different mutations have been found on more than 360 alleles. Only a few mutations are prevalent while most CBS mutations are private. The pathogenic mechanism by which CBS mutations affect enzyme activity has only been investigated in a very limited number of studies. Here we describe two common molecular mechanisms leading to CBS deficiency.

Authors have found 5 mutations, which create premature termination codons. These 5 mRNAs were supposed to be degraded by nonsense-mediated decay (NMD). This phenomenon was observed in only 2 out of 5 mutant mRNAs.

The patients carrying mutation subject to NMD were analysed in detail. The ratio of maternally and paternally derived alleles in different stages of mRNA processing was quantified. The degradation of the paternal mRNA allele started in the nucleus and was virtually completed in the cytoplasm. Obtained data suggest that alleles carrying premature termination codons are subjected to NMD probably in the later phases of mRNA processing.

Fibroblasts derived from thirteen homocystinuric patients (representing 10 different mutant alleles) contained CBS only as high molecular weight aggregates. Five of these mutants were selected for further analysis by expression in *E. coli*. The analysis revealed that these mutants were not only present as aggregates but also lack heme, a CBS ligand of unknown function. Wild type and one mutant CBS are currently being expressed as fusion protein with green fluorescent protein in CHO cells. The preliminary data confirm results from Western blot – the expression of mutant forms diffuse granular particles implying aggregosome formation. This suggests that mutation impairs CBS folding and drives the protein to aggregation in vivo.

P. Mlejnek (Department of Botany and Plant Physiology, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Brno): **The phenomenon of apoptosis in the view of the last century**.

Cell death has been neglected in contrast to other basic cellular processes such as differentiation and proliferation. Indeed, the first concept of this biological phenomenon, suggested by Virchow, considered cell death as a degenerative process produced by cell injury being akin to the post mortem autolysis of the corpse. The view of the cell death as a common part of the development in animals was established as late as in the middle of the last century (*Glücksmann, 1951*). Kerr and his co-workers consistently carried out the first systematic study of cell death, based on morphological criteria, in the early seventieth. Such an approach provided unequivocal evidence for its occurrence. Moreover, it was possible to distinguish two basic modes of cell death: necrosis and apoptosis. In the first one, apparent swelling of mitochondria is followed by a progressive dissolution of the overall cell structure including plasma and internal membrane rupture. In the second mode, condensation of the cytoplasm and chromatin is accompanied by formation of membrane-bounded bodies, in which the organelle integrity is initially maintained. A severe injury caused by toxins or ischemia usually produces necrosis whereas physiological stimuli during both embryogenesis and regulation of tissue size in postnatal life lead to cell death-exhibiting features of apoptosis. Necrosis, in contrast to apoptosis, affects groups of cells and evokes inflammation *in vivo*. Soon it was recognized that distinctive morphologic changes of nucleus and chromatin typical for apoptotic cells are related to the activation of endonucleases (*Willie and Currie* 1979). A cleavage of chromatin at the regularly spaced sites by activated endonucleases became the main biochemical hallmark of apoptosis and it is still used. Investigation of endonucleases, however, has not brought too much light on the molecular mechanisms of apoptosis. The basic framework of this process has been set at molecular level only 10 years ago (*Horvitz et al. 1994, Liu et al. 1996*). It was proved that apoptosis is manifested in two major events downstream of the cell death signals: activation of specific proteases called caspases and mitochondrial dysfunction. During apoptosis cytochrom c is released from mitochondria to cytosol. Cytosolic cytochrome c binds to Apaf-1 protein (apoptotic protease activating factor), and activates caspase-9 in the presence of ATP/dATP. This event triggers activation of caspase cascade. Caspases are family of highly conserved cysteine proteases that cleave many important cellular proteins including nuclear scaffold proteins, cytoskeletal proteins, proteins that participate in DNA repair, and many other regulatory cellular proteins. Although the relationship between protein cleavage and cell death is unclear, it is likely that collapse of cell structure and disabling of critical homeostatic functions facilitate cell dismantle. However, the matter is not as simple. Thus, it was found that caspase activation could occur upstream of cytochrome c release in some systems. In addition, examples of caspase-independent cell death can be found in many other classic apoptotic models, however, in those cases cells die more slowly and without morphological and biochemical apoptotic hallmarks. Recent studies on plants revealed that the apoptotic pathways in plants are morphologically and biochemically similar to those found in animals (*Greenberg 1996*).

Based on present knowledge it seems that cell death is essential and precisely regulated process ubiquitously occurring in all multicellular eukaryotes. However, current results indicate that cell death process is enormously complex and still only partially understood. Therefore, progress in its understanding will require further research and an open mind.

M. Kostrouchová (Laboratory of Molecular Biology and Genetics, Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University, Prague): ***Caenorhabditis elegans* – a powerful model organism for studies on gene function**.

A nematode *Caenorhabditis elegans* was chosen almost forty years ago for studies on genes involved in regulation of development and behaviour. The simple transparent body, fixed number of somatic cells, known cell lineages, the ease of growing the animal in the laboratory and short reproduction time make this organism an excellent laboratory model. An international group of scientists have chosen *C. elegans* for a genome-sequencing program that became the first finished

on a multicellular organism. *C. elegans* bioinformatics and powerful genetics and genomic multiplied the technical possibilities and allowed to address new questions. These methods include forward and reverse genetics. The forward genetics uses characteristic mutants for identification responsible genes by genetic and physical mapping. Backward genetics takes an advantage of the knowledge of the genome and targets the gene by a set of methods to elucidate its function. These methods include visualization of gene expression in time and cell specific way in transgenic lines expressing fusion proteins tagged with marker gene – lacZ or green fluorescent protein (GFP). From number of methods used for inhibition of a gene function, one proved enormously efficient and general in nature. When doing the gene inhibition by antisense RNA strategy, it was found that endogenous mRNA is degraded post transcriptionally. The whole chromosomes were analysed by RNAi technique and new functions for numerous genes were uncovered.

Authors are interested in molecular biology of nuclear hormone receptors. One of them, CHR3 is expressed in epidermis from mid embryogenesis to adulthood. The expression of CHR3 cycles during larval stages was observed. By RNAi it was shown that the receptor is critical for molting in all four larval stages. Authors found that affecting the CHR3 expression pattern is able not only to prevent the molting – shedding off the cuticle, but rather to block the complex developmental changes during larval transitions. Thus studies on CHR3 regulatory cascade is not only interesting tool for learning about gene function but may be a good strategy toward the control of pathogenic and agriculturally harmful nematode species.

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